Document Title

Summary of the toxicological and metabolism studies for fluopicolide

Part 1

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Section 5: Toxicological and metabolism studies

According to the Guidance Document SANCO/10181/2013 for applicants on preparing dossiers for the approval of a chemical active substance

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[1] It is suggested that applicants adopt a similar approach to showing revisions and version history as outlined in SANCO/10180/2013 Chapter 4, 'How to revise an Assessment Report'.

Table of Contents

CA 5 TOXICOLOGICAL AND METABOLISM STUDIES ON THE ACTIVE SUBSTANCE

Fluopicolide (AE C638206) was included in Annex I to Council Directive 91/414/EEC in 2010 (Commission Directive 2010/15/EU, Entry into Force on June 1, 2010). The expiration of approval of fluopicolide is May 31, 2023 (Commission Implementing Regulation (EU) 2017/1527). The Supplementary Dossier contains only data which were not submitted at the time of the Annex I inclusion of fluopicolide under Council Directive 91/414/EEC and which were therefore not evaluated during the first EU review. All data which were already submitted by Bayer AG (former Bayer CropScience) for the Annex I inclusion under Council Directive 91/414/EEC are contained in the Draft Assessment Report (DAR) and its Addenda, and are included in the Baseline Dossier provided by Bayer AG.

Fluopicolide is a fungicidal active substance developed by Bayer. It is the only active substance in Europe representing a class of chemistry (pyridinylmethyl-benzamides) with a unique mode of action via delocalization of a spectrin-like protein in the Oomycetes fungi.

Fluopicolide has a long track record of safe use in a large number of targeted crops within horticulture, e.g. cucumbers, lettuce and on arable crops (e.g. potato).

Fluopicolide is active against a wide range of Oomycete fungi, the causal agents of devastating plant diseases of economic importance in EU-27 such as potato late blight (*Phytophthora infestans*) or downy mildew diseases in a broad range of crops.

It provides effective, long lasting protection at low application rates against Oomycetes diseases at different stage of development of the fungi giving flexibility of use to the farmer.

Fluopicolide can be formulated with other active ingredients in different types of formulations to optimise and complete its activity.

The development of resistances of Oomycetes against existing, well-established fungicide groups represent a threat for European farmers by increasing the complexity of their plant protection programs leading to severe economic impacts. With Fluopicolide, farmers in EU-27 have access to a modern tool for their integrated crop protection programs, contributing to effective and sustainable management of resistance development and preserving high level of protection against Oomycete diseases.

By reducing the Oomycete damages, applications of Fluopicolide on target crops contribute to the achievement of optimum yield and quality, thus securing sufficient supply of high-quality potatoes and horticultural produces for European consumer destinations and markets abroad, being it fresh or for the processing industry.

Relevant information for classification as detailed in the "*Combined Draft (Renewal) Assessment Report prepared according to Regulation (EC) No 1107/2009 and Proposal for Harmonised Classification and Labelling (CLH Report) according to Regulation (EC) N° 1272/2008 – Volume 1, Level 2*" is provided in Document N1, Sections 6.1.1 – 6.1.10 and 6.2, and highlighted in light grey.

Fluopicolide was discussed at the 53rd Meeting of the Committee for Risk Assessment (RAC-53) on 11th June 2020. The CLH opinion adopted at RAC-53 concluded that fluopicolide should be classified for reproductive toxicity, category 2 (H361d). It was agreed at RAC-53 that no classification was appropriate for acute toxicity, skin or eye irritation, skin sensitisation, STOT SE, STOT RE, genotoxicity or carcinogenicity; sufficient data was available to conclude on these endpoints. It was also agreed that there should be no classification for respiratory sensitisation based on insufficient data.

Absorption, distribution, metabolism, and excretion (see Section CA 5.1)

Toxicokinetic studies on the absorption, distribution, metabolism, and excretion of fluopicolide, were conducted in the rat using two different radiolabels: [phenyl-U-^{14}C]-fluopicolide or [pyridyl-2,6-^{14}C]-fluopicolide. The major route of elimination of fluopicolide was *via* the faeces for both labels (high and low doses). No significant sex difference was observed, but higher urinary excretion was seen with the pyridyl radiolabel and repeated dosing appeared to enhance elimination *via* the urine. Tissue radioactivity levels were consistently low.

Biliary elimination was found to be a major route of excretion. At a low dose of 2 mg/kg bw, the extent of oral absorption in the bile was 94.1% for the pyridyl-labelled fluopicolide and 90.1% for the phenyl labelled fluopicolide. Oral absorption was therefore calculated to be 98.56% and 96.71% for the pyridyl and phenyl labels, respectively.

Fluopicolide was well distributed into organs and tissues and rapidly eliminated. The highest tissue residues were found in the liver and kidney.

Fluopicolide was extensively metabolised in the rat. The formation of the metabolites AE C653711 (M-01, BAM) and AE C657188 (M-02, PCA) was confirmed during the course of the biotransformation investigations and indicated that fluopicolide could be cleaved in the rat by oxidative N-alkylation of the carboxamide amine portion of the molecule. Generally, the biotransformations observed included aromatic ring hydroxylation, hydrolysis, dealkylation, acetylation, oxidative N-dealkylation and conjugation with glucuronic acid, sulphate, and glutathione. The glutathione conjugates were seen to be further metabolised by loss of glycine and glutamic acid to leave cysteine conjugates. The cysteine conjugates were seen to be further metabolised by acetylation to form the mercapturic acids or to be dealkylated and S-methylated to form S-methyl metabolites. The S-methyl metabolites were seen to be oxidised to both sulphones and sulphoxides.

Several *in vivo* single dose and an *in vivo* repeated dose study are available for the metabolite M-01 (BAM), with no significance differences in ADME behaviour being observed. The major route of elimination for M-01 was via the urine, followed by faeces with no significant sex difference observed. M-01 was extensively metabolised in the rat (biotransformations observed included aromatic ring hydroxylation, hydrolysis, decarboxylation, acetylation, and conjugation with glucuronic acid, sulphate, and glutathione).

The comparative *in-vitro* metabolism of fluopicolide was studied with CD-1 mouse, Wistar rat, Beagle dog, rabbit, and human liver microsomes. [Phenyl-U-^{14}C]-Fluopicolide was significantly metabolised by liver microsomes from all five species. A total of 10 metabolites were detected in rabbits and 8 in the other species. No human-specific fluopicolide metabolites were detected. [2,6-pyridyl-^{14}C] fluopicolide was also significantly metabolised by liver microsomes from all five species. A total of 16 metabolites were detected. No human-specific fluopicolide metabolites were detected.

Acute toxicity (see Section CA 5.2)

Fluopicolide was not acutely toxic via the oral route (LD_{50} >5000 mg/kg bw), dermal route (LD_{50} >5000 mg/kg bw) or the inhalation route (LC_{50} > 5.16 mg/L). Fluopicolide was not irritating to the skin and only slightly, transiently irritating to the eyes; there was no evidence of skin sensitisation. Furthermore, there was no indication of specific target toxicity of fluopicolide, following single exposure (STOT SE).

No classification for acute toxicity or STOT SE was therefore proposed. The outcome of the RAC-53 meeting confirms that no classification for acute toxicity or STOT-SE is required for fluopicolide.

Short-term toxicity (see Section CA 5.3)

Eight short term oral toxicity studies in mice, rats, and dogs (28-days to one-year) and one short-term dermal toxicity study in rats (28-day) are available. Decreased food consumption and body weight development were the main effects. The liver was identified as target organ in all species whilst in male rats the kidney was also affected. No adverse effects were observed after subacute exposure by the dermal route in rats up to 1,000 mg/kg bw/day.

The effects on the liver observed in rats and mice (increased weight and hepatocyte hypertrophy) are considered to be primarily adaptive responses secondary to the extensive hepatic metabolism of fluopicolide. Changes in clinical chemical parameters (cholesterol, protein, liver enzymes) were reported at higher doses indicating an influence on liver function. Hepatocytic necrosis was observed at ≥ 3,200 ppm in mice. The dog was less sensitive than the rodents and only showed slight liver effects.

Kidney effects were only observed in male rats and were characterised by an increase in the severity and incidence of accumulation of hyaline droplets in the proximal tubule, accompanied by single cell death in the proximal tubule epithelium, foci of basophilic (regenerating) tubules, granular casts and increased relative kidney weights at higher dose levels in the sub chronic study only. Hyaline droplets have been recognized as indicator of changes associated with the accumulation of $\alpha 2\mu$-globulin which is male rat specific and therefore not relevant to humans.

The lowest NOAEL was derived from the 90-day rat study at 100 ppm (equivalent to 7.4/8.4 mg/kg bw/day (M/F)), increases in cholesterol plasma levels and increased relative liver and kidney weights with associated histopathological findings were seen at the LOAEL.

No consistent change indicative of severe organ dysfunction was seen in a dose range relevant for STOT-RE classification. Therefore, no classification was proposed. The outcome of the RAC-53 meeting confirms that no classification for STOT-RE is required for fluopicolide.

Genotoxicity (see Section CA 5.4)

Fluopicolide has been tested in a battery of genotoxicity tests, both *in vitro* (six Ames tests, two chromosomal aberration assays in Chinese hamster V79 cells and human lymphocytes and one HPRT mutation assay in Chinese hamster V79 cells) and *in vivo* (three mouse micronucleus assays, one UDS assay and one Comet assay).

A very slight increase in the number of revertant colonies in strain TA 98 (+S9) and TA 1537 (+S9) was seen at the highest (precipitative) concentration of 5000 µg/plate in one of the Ames tests. No evidence of mutagenic activity was observed in four additional bacterial reverse mutation assays, and a recently conducted Ames test was also negative and confirmed the overall negative outcome. Fluopicolide was not mutagenic in mammalian cells (Chinese hamster V79 cells) and a recently conducted Comet assay confirmed that fluopicolide was not mutagenic *in vivo*.

In a chromosomal aberration assay *in vitro* (Chinese hamster V79 cells), an increase of aberrant cells occurred at cytotoxic concentrations where mitotic indices were clearly below the limit of 50% (doubtful biological significance). A clear negative response was seen in a subsequent chromosome aberration assay in human lymphocytes. Furthermore, the negative result was confirmed *in vivo* in two mouse micronucleus assays (up to the limit dose of 2000 mg/kg bw). As the ratio of polychromatic to normochromatic erythrocytes was not significantly affected and no clinical signs were observed in either assay, a third assay was performed in mice by the intraperitoneal route. This assay gave a clear negative result for clastogenicity *in vivo* at dose levels showing clear cytotoxicity of the bone marrow.

Altogether these findings clearly show that fluopicolide is devoid of any genotoxic potential when tested *in vitro* and *in vivo*.

Therefore, no classification for genotoxicity was proposed. The outcome of the RAC-53 meeting confirmed that no classification for genotoxicity was warranted for fluopicolide.

Long-term toxicity and carcinogenicity (see Section CA 5.5)

A long-term combined toxicity and carcinogenicity study in rats (2-years) and an oncogenicity study in mice (18-months) are available).

Rats

The liver and kidneys were the main target organs characterised by increased weights and histopathological findings. Secondary to the increased metabolic activity of the liver, an increased incidence of cystic follicular cell hyperplasia in the thyroids of males was recorded. The No Observed Adverse Effect Level (NOAEL) for toxicity was 200 ppm in both males and females (equivalent to 8.4 and 10.8 mg/kg bw/day in males and females, respectively). Furthermore, there was no evidence of carcinogenicity with fluopicolide up to and including the dose level of 2,500 ppm (equivalent to 109.4 and 142.2 mg/kg bw/day, in males and females, respectively).

Mice

In mice, severe reductions in body weight gain were seen at 3,200 ppm indicating that the Maximal Tolerated Dose (MTD) was reached. The target organ identified was the liver. Higher liver weights, enlarged liver, increased number of masses and nodules in the liver were observed at 400 and 3,200 ppm at 52 and 78 weeks. These changes were associated with hepatocellular hypertrophy at 52 and 78 weeks, and high incidence of altered cell foci at 3,200 ppm at 78 weeks. A high incidence of hepatocellular adenoma was observed at 3,200 ppm at 78 weeks in both males and females and to a lesser extent at 52 weeks in females. Therefore, the NOAELs are 50 ppm for toxicity (equivalent to 7.9 mg/kg bw/day and 11.5 mg/kg bw/day in males and females, respectively) and 400 ppm for carcinogenicity (equivalent to 64.5 mg/kg bw/day and 91.9 mg/kg bw/day in males and females, respectively). These benign liver tumours occurred only at the highest dose reaching the MTD (severe body weight gain reduction in high dose animals). Moreover, no tumours were observed in other mouse tissues and these tumours did not progress into malignant neoplasia during the lifespan of these animals, no increased incidence of hepatocellular carcinoma was observed in any groups after a 78-week treatment period. The mode of action for the increased incidence of liver adenomas was found to be CAR-mediated and thus, secondary to liver enzyme induction, like that of phenobarbital (see section CA 5.8.2 of this document). This MOA is considered of no relevance in humans.

No classification for carcinogenicity was therefore proposed. The outcome of the RAC-53 meeting confirmed that no classification for carcinogenicity was warranted for fluopicolide.

Reproductive toxicity (see Section CA 5.6)

Fluopicolide has been investigated in a two-generation reproductive toxicity study in rats (including preliminary study) and in developmental toxicity studies (including preliminary studies) in rats and rabbits.

Generational studies

In the preliminary study for the two-generation study, general toxicity was seen at 2500 ppm characterised by bodyweight gain reductions in F_0 males (pre-mating), F_0 females (gestation and lactation) and F_1 offspring. Reproductive parameters were considered to be unaffected by treatment with fluopicolide.

In the main 2-generation study, body weight gain and food consumption were generally low for adult animals treated at 2,000 ppm throughout the study. Oestrus cycling, mating performance, fertility and fecundity were not affected. Gestation length, parturition process and sperm parameters were also unaffected by treatment and sexual maturation (age and bodyweight at the time of attainment of vaginal opening or balano-preputial separation), were not affected by treatment. Litter parameters at birth of the F_1 and F_2 progeny and their survival to weaning showed no detrimental effects of treatment. Body weights were significantly reduced in male and female offspring at 2,000 ppm from Day 14 through to weaning, secondary to direct consumption of the test diet.

The liver and kidneys were identified as target organs in adult animals (increased weights and histopathological correlates). The liver findings are considered to be an adaptive change and not a treatment related effect.

In conclusion, a dietary concentration of fluopicolide at 500 ppm should be considered as the No Observed Adverse Effect Level (NOAEL). The minimum mean achieved dosages for the F_0 animals at this NOAEL (500 ppm) are 25.5 mg/kg bw/day for the males and 32.9 mg/kg bw/day for the females. The No Observed Effect Level (NOEL) for developing offspring is considered to be 500 ppm, equivalent to a minimum mean achieved dosage of 35.8 mg/kg bw/day for F_0 females during gestation and lactation based on the decreased bodyweight gain at 2,000 ppm. The NOEL for reproductive parameters is considered to be 2,000 ppm, equivalent to a mean achieved dosage of at least 103.4 mg/kg bw/day for F_0 males and at least 127.3 mg/kg bw/day for F_0 females before pairing.

Developmental studies

Maternal findings in the preliminary rat study comprised reduced body weight gain and food consumption at 1,000 mg/kg bw/day. There were no findings at necropsy. Post-implantation loss was elevated at 1,000 mg/kg bw/day and included one total resorption. Mean foetal weight and crown-rump length were reduced at 1000 and 500 mg/kg bw/day.

In the main rat developmental study, body weights and weight gains were decreased in the animals from the high dose group (particularly during the sensitive period of GD 7-10).

Mean foetal body weights, crown-rump lengths and placental weights were slightly, but statistically significantly decreased in the high dose group. Litter size, number of live and dead foetuses, sex ratios and incidences of early and late resorption were unaffected treatment.

Morphological examination of the foetuses revealed one foetus with multiple malformations at the vertebral column and pelvis in the intermediate dose group and one foetus with microphthalmia in the high dose group. These findings are considered to be incidental due to their isolated occurrence.

Foetuses from the high dose group showed increased incidences of minor skeletal defects at the thoracic vertebrae, sternebrae and ribs. However, only a small number of foetuses in single litters was affected and these findings are not considered to have adverse consequences for the foetuses in postnatal life. The observations represent mostly a perturbation of ossification, transient in nature, being resolved as ossification progresses. In addition, a delayed ossification was detected at 700 mg/kg bw/day which indicated together with the decreased foetal weight and length a generally retarded foetal development at this maternally toxic dose level. Fluopicolide was not teratogenic in this developmental toxicity study in rats and the No Observed Effect Level (NOEL) is 60 mg/kg bw/day for maternal toxicity and for developmental toxicity.

In a rabbit range finding study, animals from the 100, 250, 500 or 1,000 mg/kg bw/day group were found dead, killed moribund or killed after abortion up to Day 23 of the study preceded by decreases in food consumption and body weight gains. Embryo foetal development was not affected.

In the main rabbit developmental study, three animals of the high dose group were found dead and 15 animals of this group were killed after premature delivery from Day 22-29 of gestation, preceded by clinical signs and decreased body weight gain and food consumption. Necropsy findings in the stomach, uterus and liver were observed.

Dead foetuses were present in most premature deliveries. Mean foetal body weights, crown-rump lengths and placental weights were decreased in the animals from the high dose group. Litter size, number of live and dead foetuses, sex ratios and incidences of early and late resorption remained were not affected. No teratogenic effects were observed in the foetuses at any dose level.

Fluopicolide did not cause any maternal toxicity or embryotoxicity at the dose of 20 mg/kg bw/day. Therefore, the NOAEL is 20 mg/kg bw/day for maternal toxicity and for developmental toxicity.

It was proposed that fluopicolide should not be classified as a reproductive toxicant based on the ECHA Guidance on the application of the CLP criteria. However, at the RAC-53 meeting it was concluded that fluopicolide should be classified for reproductive toxicity, category 2 (H361d), based on the observed increase in minor skeletal findings in the rat developmental toxicity study.

Neurotoxicity studies (see Section CA 5.7)

Preliminary acute, definitive acute and subchronic neurotoxicity studies in rats are available.

In the acute study, no animals died, no signs were seen at routine observations and body weights, food consumption, food conversion efficiency and brain weights and dimensions were unaffected by treatment. Macropathological and histopathological examination of the tissues did not reveal any findings related to the administration of fluopicolide. At the neurobehavioral screening, the only treatment-related finding were low body temperatures recorded in animals receiving 2,000 mg/kg bw at Day 1 only. Therefore, signs of a direct neurotoxic potential were not evident in this study. The No Observed Effect Level (NOEL) on this study is considered to be 100 mg/kg bw.

In a subchronic study, no animals died during the study. Routine daily clinical signs, detailed weekly observations, FOB and motor activity assessments, brain weights (absolute) and measurements, and neuropathology were unaffected by the test substance. Only adaptive changes occurred in the liver at 1,400 and 10,000 ppm and male-specific nephropathy occurred at 1,400 and 10,000 ppm. Thus, it is concluded that the administration fluopicolide to CD rats for 13 weeks via the diet at concentrations of up to 10,000 ppm (equivalent to 781 mg/kg bw/day in males and 866 mg/kg bw/day in females) did not result in any neurotoxicity. The No Observed Effect Level (NOEL) in this study was established at 200 ppm (equivalent to 15.0 mg/kg bw/day in males and 18.0 mg/kg bw/day in females).

No studies on delayed polyneuropathy were conducted as they were not necessary.

Toxicity studies on metabolites (see Section CA 5.8.1 in Part 2 of this document)

Toxicological studies have been conducted on the metabolites M-01 (AE C653711, BAM), M-02 (AE C657188, PCA), M-04 (AE C657378), M-05 (AE 1344122), M-09 (AE B102859), M-10 (AE 1344123), M-14 (AE 1388273) and M-15 (AE 1413903).

Overall, it was concluded that none of the metabolites were genotoxic; furthermore, all metabolites were less toxic than the parent fluopicolide, with the exception of M-01, which is of similar toxicity. An almost complete data set of M-01 demonstrated that M-01 is toxicological not relevant.

Owing to the recent classification of fluopicolide as toxic to reproduction, category 2 (H361d) at the 53rd meeting of the Committee for Risk Assessment (RAC-53), further studies will be conducted (after alignment with the RMS) to demonstrate that the leaching metabolites do not share the properties of the parent.

Mechanistic studies (see Section CA 5.8.2 in Part 2 of this document)

In order to demonstrate the mode of action for the observed increase in the incidence of hepatocellular adenoma in mice, a series of *in vitro* studies and one *in vivo* study were conducted.

In vitro studies conducted in cultured male and female C57BL/6 mouse, CarKO/PxrKO mouse and human hepatocytes confirmed a rodent specific CAR/PXR mode of action. These studies demonstrated that significant CAR and PXR activation and hepatocyte proliferation was induced in wild type hepatocytes but not in CarKO/PxrKO or human hepatocytes.

In a 28-day explanatory toxicity study, fluopicolide was shown to be a strong inducer of total cytochrome P450 and BROD and PROD associated activities. In addition, fluopicolide produced a marked transient liver cell proliferation on Day 7 which returned to control levels on Day 28. These findings were similar to those observed with phenobarbital showing that fluopicolide is a phenobarbital-like compound.

This mechanism of action is clearly specific to the mouse and of no relevance to humans.

CA 5.1 Studies on absorption, distribution, metabolism and excretion in mammals

In vivo studies

Toxicokinetic studies on the absorption, distribution, metabolism, and excretion of fluopicolide were conducted in the rat. Studies were performed using two different radiolabels: [phenyl-U-^{14}C]-fluopicolide or [pyridyl-2,6-^{14}C]-fluopicolide.

The major route of elimination of fluopicolide was via the faeces for both the 10 and 100 mg/kg bw oral dose and for both pyridyl (69 to 72% of the administered dose) and phenyl (82 to 88%) ring radiolabels. No significant sex difference was observed. There was a tendency towards a higher urinary excretion level with the pyridyl radiolabel (19% in males and 22% in females for the 10 mg/kg bw dose) compared with the phenyl radiolabel (10% in males and 13% in females for the 10 mg/kg bw dose). This suggests that a proportion of the metabolites that were formed differed between the two radiolabels and were presumably linked to the formation of AE C657188 (M-02, PCA) from the pyridyl ring moiety and AE C653711 (M-01, BAM) from the phenyl ring.

Following repeated (14x) daily oral administrations of [phenyl-U-^{14}C]-fluopicolide the total recovery of radioactivity was approx. 96% of the administered dose, with the faeces, again, being found to be the major route of elimination representing 79% for the males and 72% for the females. The urine was found to represent 15% of the administered dose for the males and 21% for the females. It appeared that repeated dosing enhanced elimination via urine compared with the single oral dose.

Tissue radioactivity levels were consistently low and ranged between 0.46 to 1.25% of the administered dose for the single dose studies and a mean of 0.38% for the repeat dose study.

Investigations in bile-cannulated rats over 48 hours showed a large proportion of the radioactivity found in the faeces had been absorbed and then eliminated via the bile. The extent of oral absorption based on the biliary excretion study only, for the 10 mg/kg bw oral dose, was 80% of the administered dose for the phenyl radiolabel and 62% for the pyridyl radiolabel. However, blood and plasma pharmacokinetic data show the systemic exposure was similar between both the radiolabels and the sexes. The bioavailability of fluopicolide, considering the material undergoing entero-hepatic recirculation, was calculated to be 75 to 88% of the administered dose. In more recent studies conducted with [pyridyl-2,6-^{14}C]-fluopicolide and [phenyl-U-^{14}C]-fluopicolide in bile cannulated rats over 48 hours, at a low dose of 2 mg/kg bw, the extent of oral absorption in the bile was 94.1% for the pyridyl-labelled fluopicolide and 90.1% for the phenyl labelled fluopicolide. Absorption was therefore calculated to be 98.56% and 96.71% respectively for these studies.

Recovery in all four studies was good. However, the newly conducted studies are considered more reliable as they contain analytical data which demonstrates that the derived absorption values are accurate (this data was lacking in the previous studies). Therefore, the oral absorption value to be used in the risk assessment is derived from the recently conducted bile excretion studies. The table below summarises the available data.

Table 5.1- 1: Biliary excretion and oral absorption for [Phenyl-U-14C]-AE C638206 and [Pyridyl-2,6-14C]-AE C638206

Reference	Label	Dose	Biliary excretion	Calculated absorption
[redacted] 2002; M-212243-01-1	[Phenyl-U-^{14}C]-AE C638206	10 mg/kg bw/d	Males: 77% Females: 80%	Mean: 86%* (84/88% in M/F)
[redacted] 2003; M-230976-01-1	[Pyridyl-2,6-^{14}C]-AE C638206	10 mg/kg bw/d	Males: 59% Females: 64%	Mean: 76.5%* (75/78% in M/F)
[redacted] 2020; M-681576-01-1	[Pyridyl-2,6-^{14}C]-AE C638206	2 mg/kg bw/d	94.1%	98.5%
[redacted] 2020; M-681498-01-1	[Phenyl-U-^{14}C]-AE C638206	2 mg/kg bw/d	90.1%	96.71%

The oral absorption values form the new studies are 98.5% and 96.71% for [Pyridyl-2,6-14C]-AE C638206 and [Phenyl-U-^{14}C]-AE C638206, respectively; as a conservative approach, the lower of the 2 values will be taken forward for the risk assessment. Therefore, the oral absorption of fluopicolide is calculated to be 96.71%.

Fluopicolide was well distributed into organs and tissues (blood T_{max} 5.5 to 7.5 hours and plasma T_{max} 6.5 to 8 hours for 10 mg/kg bw) followed by a moderately rapid elimination such that the majority was eliminated by 48 hours post dose followed by a slower terminal elimination phase with a mean half-life of approx. 99 hours for blood. A lower mean half-life of 16 hours was observed for plasma due to the difference in limits of quantification.

The highest tissue residues were found in the liver and kidney and (to a lesser extent) in the spleen and blood.

In tissue kinetic studies the highest tissue concentrations were observed in the intestine and contents, reflecting a combination of unabsorbed material and biliary excretion. The next highest concentrations were consistently observed in the liver, kidneys, and adrenals albeit that the concentrations were decreasing with time post dosing. AE C653711 (M-01, BAM), AE 0717559, (no metabolite number assigned) AE C643890 (M-06) and AE 0717560 (no metabolite number assigned) were identified in the liver 8 hour post dosing with [phenyl-U-^{14}C]-fluopicolide.

Fluopicolide was extensively metabolised in the rat. The formation of the metabolites AE C653711 (M-01, BAM) and AE C657188 (M-02, PCA) was confirmed during the course of the biotransformation investigations and indicated that fluopicolide could be cleaved in the rat by oxidative N-alkylation of the carboxamide amine portion of the molecule. Generally, the biotransformations observed included aromatic ring hydroxylation, hydrolysis, dealkylation, acetylation, oxidative N-dealkylation and conjugation with glucuronic acid, sulphate, and glutathione. The glutathione conjugates were seen to be further metabolised by loss of glycine and glutamic acid to leave cysteine conjugates. The cysteine conjugates were seen to be further metabolised by acetylation to form the mercapturic acids or to be dealkylated and S-methylated to form S-methyl metabolites. The S-methyl metabolites were seen to be oxidised to both sulphones and sulphoxides.

Several *in vivo* single dose and an *in vivo* repeated dose study are available for the metabolite M-01 (BAM), with no significance differences in ADME behaviour being observed. Following a single oral low (10 mg/kg bw) and high (150 mg/kg bw) dose of M-01 (BAM), the major route of elimination was via the urine (79 to 84% of the administered dose), followed by faeces (12 to 14% of dose). Similarly, following repeated oral dosing of [phenyl-U-^{14}C]-M-01 urine was the major route of elimination, comprising 77 to 82% of the administered dose (faeces constituted 16 to 19% of the dose). No significant sex difference was observed. Overall, the residue concentration in tissues was higher in male rats (residues in the testes were also higher than those in the ovaries or uterus). Tissue radioactivity levels were consistently low (1.17 to 2.21% of the administered dose for the single dose studies and 0.59 to 1.07% for the repeat dose study), with the highest tissue residues in the skin and fur, liver, and kidneys.

M-01 was extensively metabolised in the rat. Generally, the biotransformations observed included aromatic ring hydroxylation, hydrolysis, decarboxylation, acetylation, and conjugation with glucuronic acid, sulphate, and glutathione. Glutathione conjugates were further metabolised by loss of glycine and glutamic acid to leave cysteine conjugates. The cysteine conjugates were further metabolised by acetylation to form the mercapturic acids or dealkylated and S-methylated to form S-methyl metabolites. The most abundant metabolite detected was a mercapturic acid conjugate of hydroxy-chlorobenzamide (15 to 26% of dose) detected in urine.

***In vitro* studies**

The comparative *in-vitro* metabolism of fluopicolide was studied with liver microsomes from CD-1 mouse, Wistar rat, Beagle dog and human, and, in a later study, from New Zealand White rabbits. Incubations were performed with [phenyl-U-^{14}C]-fluopicolide and [2,6-pyridyl-^{14}C] fluopicolide at two concentrations (1 and 10 µM) at 1, 60 and 120 minutes.

[Phenyl-U-^{14}C]-Fluopicolide was significantly metabolised by liver microsomes from all five species. Conversion of fluopicolide was 98% in dog, 82% in mouse, 68% in human, 98% in rabbit and 54% in rat microsomes after 120 minutes of incubation. A total of 8 metabolites were detected, named Metabolite 1 to 8 based on their HPLC retention time. In the rabbit, a total of 10 metabolites were detected. Metabolites accounting for ≥ 5% were considered as main metabolites. Overall, in the first study, five main metabolites were detected: Metabolite 1 (mouse, rat and human) and Metabolite 2 (mouse and rat), Metabolite 3 and Metabolite 5 (mouse, dog and human) and Metabolite 6 which was detected as a main metabolite in the four species. Metabolite 2 was detected in the mouse and rat microsome incubations only. In the second study (rabbit only) Metabolites 2, 7, 9 & 10 were considered as main metabolites; these corresponded to metabolites 1, 3, 5 and 6 in the first study. Four metabolites (designated M1, M3, M5 and M6 in the second study) were unique to the rabbit. No human-specific fluopicolide metabolites were detected.

[2,6-pyridyl-^{14}C] fluopicolide was also significantly metabolised by liver microsomes from all five species. Conversion of fluopicolide was 93% in rabbit, 92% in mouse, 72% in human, 67% in rat and 59% in dog liver microsomes after 120 minutes of incubation. A total of 16 metabolites were detected. Metabolites accounting for ≥ 5% were considered as main metabolites. Overall, five main metabolites were detected (M6, M9, M10, M13 and M14). No human-specific fluopicolide metabolites were detected.

A proposed metabolic pathway for fluopicolide in the rat is presented in Figure 5.1.1, 5.1.1a and 5.1.1b below:

Figure 5.1- 1: Proposed metabolic pathway for fluopicolide in the rat

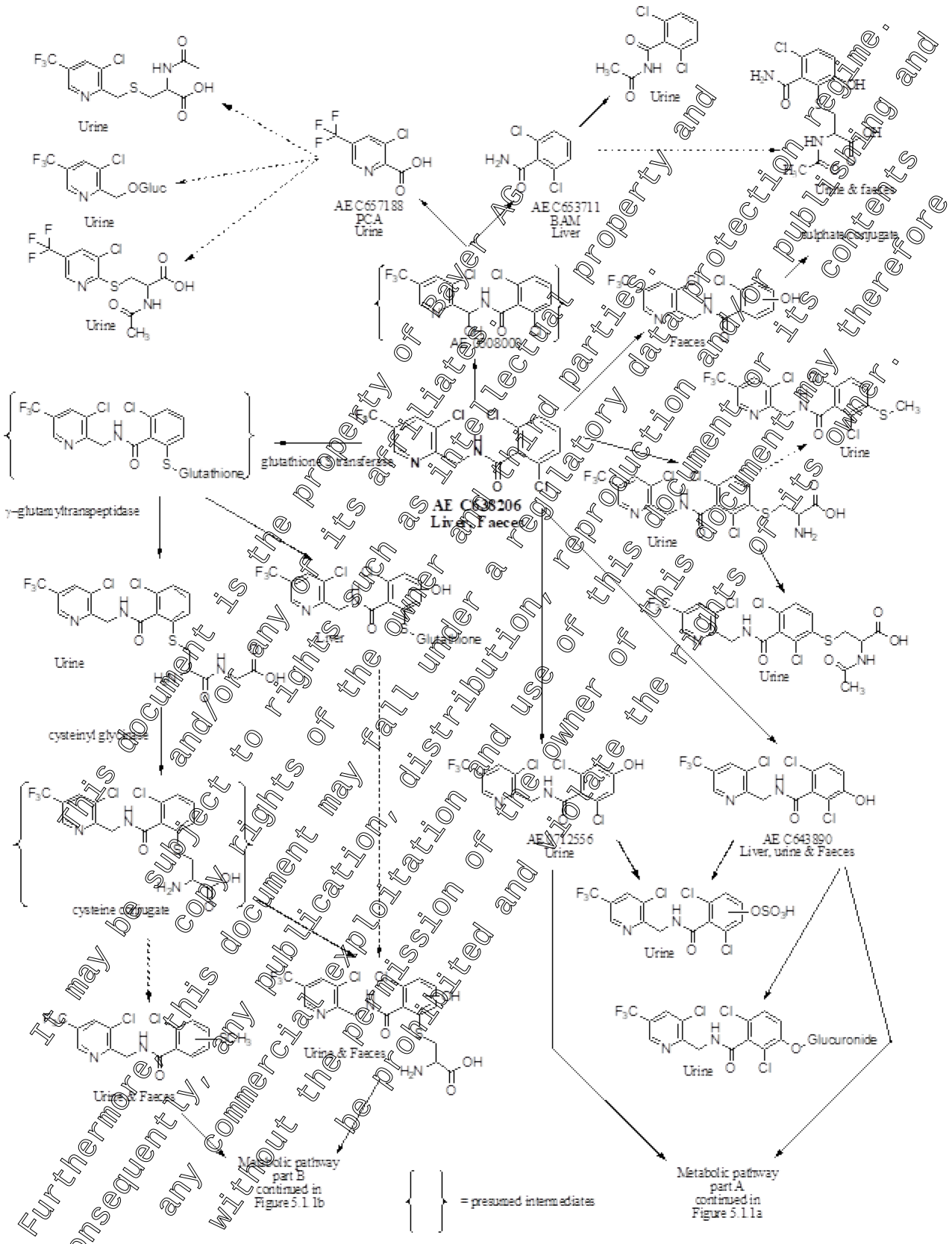


Figure 5.1- 1a: Proposed metabolic pathway for fluopicolide in the rat (cont.)

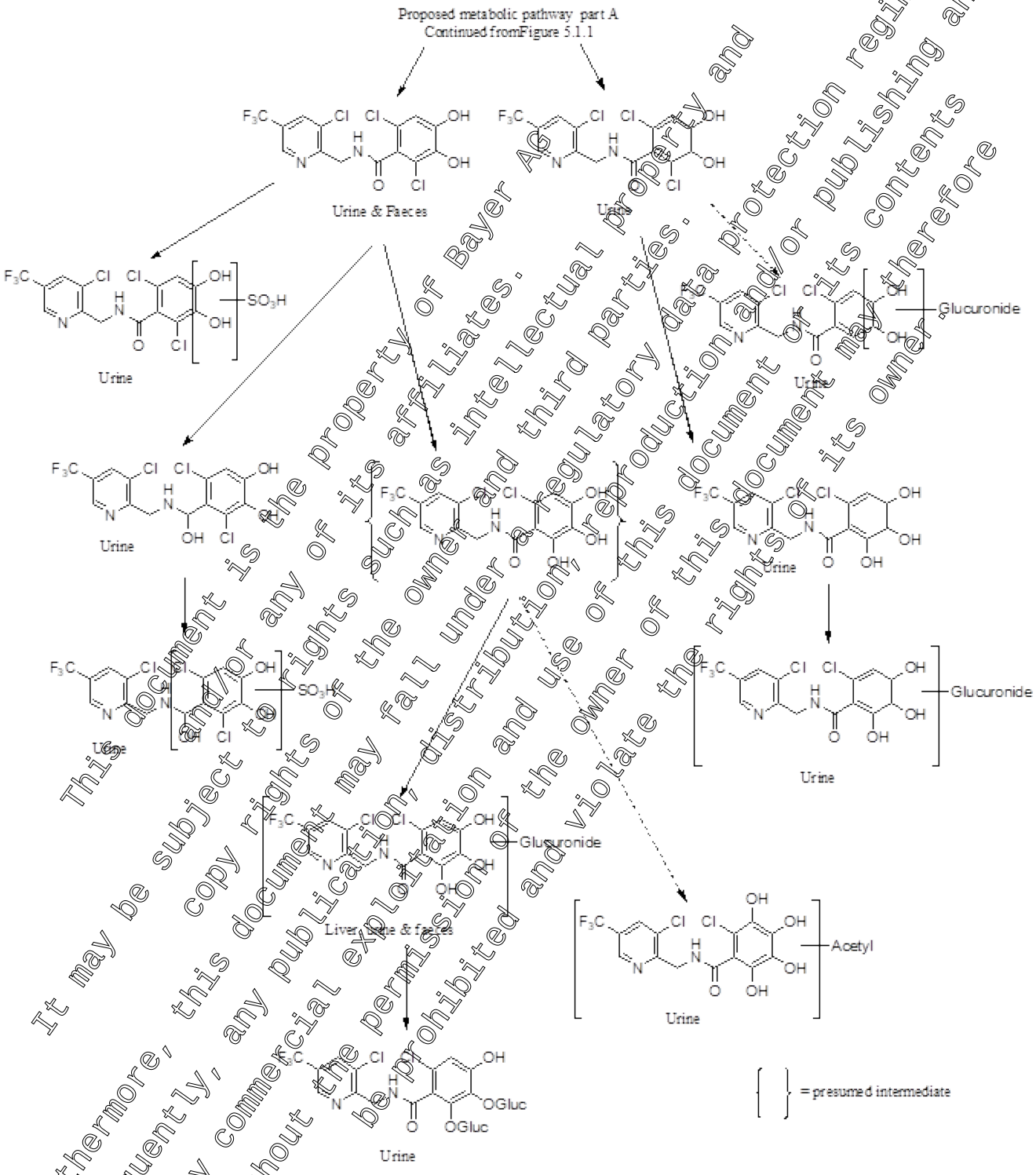


Figure 5.1- 1b: Proposed metabolic pathway for fluopicolide in the rat (cont.)

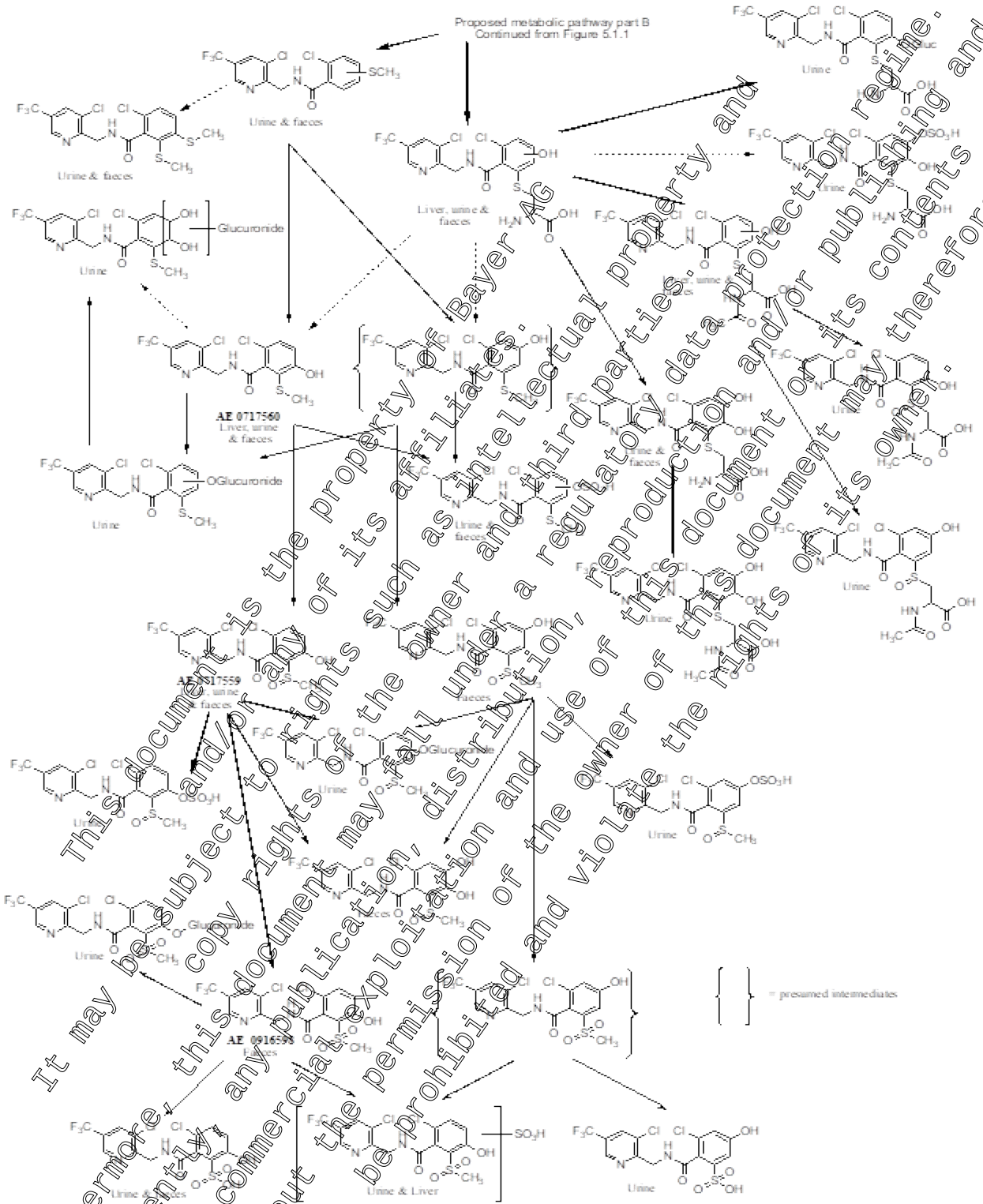


Table 5.1- 2: Summary of toxicokinetic studies

Method Guideline GLP compliance	Test Conditions	Result	Reference
Combined ADME & kinetics preliminary study Non guideline preliminary study GLP	[Phenyl-U-^{14}C]-Fluopicolide ADME: 25 mg/kg bw, 2/sex; 500 mg/kg bw, 2/sex. Blood kinetic: 25 mg/kg bw, 2/sex; 500 mg/kg bw, 2/sex. [Pyridyl-2,6-^{14}C]-fluopicolide ADME: 25 mg/kg bw, 2/sex. Blood kinetic: 25 mg/kg bw, 2/sex.	Rapidly adsorped Blood C_{max} between 8 to 12 hours. At low dose residues were below 0.10 µg/g in all tissues except the liver, kidney and blood, 168 h after dosing. The major metabolic reactions identified were aromatic hydroxylation of the phenyl ring, glucuronidation of the phase I hydroxyl products and sequential metabolism through the mercapturic acid pathway.	[redacted] 2000; M-197858-01-1 KCA 5.1.1/01
A.D.E. study, rat USEPA OPPTS 870.7485 JMAF 59 NohSan No 4200 GLP	[Phenyl-U-^{14}C]-AE C638206 Single high & low dose	Faeces: 82% to 88% of administered dose. Urine: 9% to 13% of administered dose. Almost complete excretion occurred within: 48 hours (low dose) 24 hours (high dose) Tissues: 0.75% to 1.25% of administered dose at 168 hours post-dose: Highest residues in liver & kidneys (both sexes/doses) & skin & fur (high dose females)	[redacted] 2001; M-204784-01-1 KCA 5.1.1/02
A.D.E. study, rats USEPA OPPTS 870.7485 JMAF 59 NohSan No 4200 GLP	[Pyridyl-2,6 -^{14}C]-AE C638206 Single oral low dose rat	Excretion almost complete within 48 hours Urine: 21% to 27% of administered dose Faeces: 69-72% of administered dose Tissues: 0.7% to 0.5% of administered dose at 168h post-dose Highest residues in liver, kidneys and blood	[redacted] 2001; M-202609-02-1 KCA 5.1.1/03

Method Guideline GLP compliance	Test Conditions	Result	Reference
Rat bile excretion study USEPA OPPTS 870.7485 JMAF 59 NohSan No 4200 GLP	[Phenyl-U-^{14}C]-AE C638206 4 male & 4 female bile-duct cannulated rats at 10 mg/kg bw; 4 male & 4 female bile-duct cannulated rats at 100 mg/kg bw.	Biliary elimination Low dose: 77% (males), 83% (females) 80% (mean) detected in the bile of cannulated rats. High dose: 34% (males) 41% (females), 37% (mean) *Absorption saturated at the high dose.*	[redacted] 2002; M-212243-01-1 KCA 5.1.1/04
Rat bile excretion study USEPA OPPTS 870.7485 JMAF 59 NohSan No 4200 GLP	[Pyridyl-2,6-^{14}C]-AE C638206 Single oral low dose	Biliary elimination: 59% (males), 64% (females), 62% (mean) detected in the bile of cannulated rats.	[redacted] 2003; M-230976-01-1 KCA 5.1.1/05
Rat blood and plasma kinetics study EU (=EEC): 87/302/EEC; JMAF: 59, Nohsan No 4200; USEPA (=EPA): OPPTS 870.7485 GLP	[Pyridyl-2,6-^{14}C]-AE C638206 [Phenyl-U-^{14}C]-AE C638206 10 mg/kg bw: 4/sex 100 mg/kg bw: 4/sex Single oral gavage dose	Maximal concentration in blood or plasma: 2 µg equivalents (after 6-8h; low dose) 7 µg equivalents (after 8-12; high dose) Terminal elimination half-life was 100 h form whole blood Total systemic exposure was 50 µg.h/g (low dose) or 300 µg.h/g (high dose) Exposure was not dose proportional (lower proportion absorbed at the high dose)	[redacted] Vinck, K.; 2003; M-221902-01-1 KCA 5.1.1/06
Rat tissue kinetics study USEPA OPPTS 870.7485 JMAF 59 NohSan No 4200 GLP	[Phenyl-U-^{14}C]-AE C638206 10 mg/kg bw:16/sex 100 mg/kg bw: 16/sex Sacrificed at 8, 24 or 30, 36 or 48, 72 (males) & 120 hours (females).	Rapidly absorbed Maximal tissue concentrations achieved 8 hours post dose at 10 and 100 mg/kg bw. *Pharmacokinetic profiles similar between sexes.* Liver metabolites 12 metabolites and parent material found in liver. Metabolites identified were AE C653711 (M-01), AE 0717559 (no M number assigned), AE C643890 (M-06), and AE 0717560 (no M-number assigned).	[redacted] 2003; M-221892-01-1 KCA 5.1.1/07

Method Guideline GLP compliance	Test Conditions	Result	Reference
Rat tissue kinetic study EU (=EEC): 94/79/EC; JMAF: 12, Nousan No 8147; USEPA (=EPA): OPPTS 870.7485 GLP	[Pyridyl-U-^{14}C]-Fluopicolide 10 mg/kg bw: 16/sex Sacrificed at 6 or 7, 24, 36, 48 (males) & 120 hours (females).	Distributed into tissues, followed by a significant and rapid decrease in tissue concentrations. *No sex differences.* Highest radioactivity concentrations were in the intestine and contents The next highest concentrations were in the liver, kidneys, adrenals and cardiac blood which declined with time post dose.	[redacted] 2003; M-221885-01-1 KCA 5.1.1/08
Rat metabolism USEPA OPPTS 870.7485 JMAF 59 NohSan No 4200 GLP	[Pyridyl-2,6-14C]-AE C638206: Single oral low dose	Extensively metabolised Urine: 17% to 21% of administered dose, up to 28 metabolites Faeces: 63% of administered dose, 31 metabolites	[redacted] 2004; M-227023-02-1 KCA 5.1.1/09
Rat metabolism USEPA OPPTS 870.7485 JMAF 59 NohSan No 4200 GLP	[Phenyl-U-^{14}C]-AE C638206: Single oral high dose	Extensively metabolised Urine: 4% to 6% of administered dose, 46 metabolites detected Faeces: 86% to 87% of administered dose, 14 metabolites detected	[redacted] 2004; M-227025-02-1 KCA 5.1.1/10
Rat metabolism (Plus, Amendment No. 1) USEPA OPPTS 870.7485 JMAF 59 NohSan No 4200 GLP	[Phenyl-U-^{14}C]-AE C638206 Single oral low dose	Extensively metabolised Urine: 9% to 13% of administered dose, up to 55 metabolites Faeces: 81% to 82% of administered dose, up to 52 metabolites	[redacted] 2004; M-227026-02-1 KCA 5.1.1/11
A.D.M.E. study, rat USEPA OPPTS 870.7485 JMAF 59 NohSan No 4200 GLP	[Phenyl-U-14C]-AE C638206 Repeat oral low dose	No evidence of accumulation, extensively metabolised Urine: 15% to 22% of administered dose, 46 metabolites detected Faeces: 73% to 79% of administered dose, 14 metabolites detected Tissues: 0.38% of administered dose Highest residues in liver, kidneys and blood	[redacted] 2004; M-227027-02-1 KCA 5.1.1/12

Method Guideline GLP compliance	Test Conditions	Result	Reference
Evaluation of oral bioavailability in the rat	Not applicable	Evaluation of the oral absorption from the bile excretion studies. A bioavailability of 74% is concluded to be a conservative estimate	[redacted] 2007 M-287367-01-1 KCA 5.1.1/13
[Phenyl-U-^{14}C]-M-01: Single oral high dose A.D.M.E study in the rat USEPA OPPTS 870.7485 JMAF 59 NohSan No 4200 GLP	[Phenyl-U-^{14}C]-M-01: 4 male & 4 female rats at 150 mg/kg bw.	The major elimination route was via urine (79 to 84% of dose including cage washes), while faeces contained 12 to 13% of dose. Excretion of radioactivity was relatively slow, with 96 hours required for close to complete elimination via urine. At 168 hours post dose, tissues residues were low (≤ 1.2% of dose). Skin and fur contained the highest residues, with liver and kidneys containing the next highest residues. [Phenyl-U-^{14}C]-M-01 was extensively metabolised in high dose rats (150 mg/kg bw/day) with up to 20 metabolites in urine and 12 in faecal extracts. The same routes of metabolism as seen in the low dose group were observed in high dose animals. The most abundant metabolite detected was a mercapturic acid conjugate of hydroxy-chlorobenzamide (18 to 21% of dose).	[redacted] 2003 M-218352-01-1 KCA 5.1.1/14

Method Guideline GLP compliance	Test Conditions	Result	Reference
[Phenyl-U-^{14}C]-M-01: Single oral low dose A.D.M.E study in the rat USEPA OPPTS 870.7485 JMAF 59 NohSan No 4200 GLP	[Phenyl-U-^{14}C]-M-01: 4 male & 4 female rats at 10 mg/kg bw	The major elimination route was via urine (81 to 84% of dose including cage washes), while faeces contained 12 to 14% of dose. Excretion of radioactivity was relatively slow, with 96 hours required for close to complete elimination via urine. At 144 hours post dose, tissues residues were low (≤ 2.2% of the dose). Liver and kidneys (organs of excretion and metabolism) contained the highest residues. [Phenyl-U-^{14}C]-M-01 was extensively metabolised in low dose rats (10 mg/kg bw/day) with up to 14 metabolites in urine and 6 in faecal extracts. Biotransformations observed included aromatic ring hydroxylation, hydrolysis, decarboxylation, acetylation, and conjugation with glucuronic acid, sulfate, and glutathione. Glutathione conjugates were further metabolised by loss of glycine and glutamic acid to leave cysteine conjugates. The cysteine conjugates were further metabolised by acetylation to form the mercapturic acids or to dealkylated and S-methylated to form S-methyl metabolites. The most abundant metabolite detected was a mercapturic acid conjugate of hydroxy-chlorobenzamide (25 to 26% of dose)	[redacted] 2003; M-218350-01-1 KCA 5.1.1/15

Method Guideline GLP compliance	Test Conditions	Result	Reference
[Phenyl-U-^{14}C]-M-01: Repeat oral low dose A.D.M.E study in the rat USEPA OPPTS 870.7485 JMAF 59 NohSan No 4200 GLP	[Phenyl-U-^{14}C]-M-01: 5 male & 5 female rats at 10 mg/kg bw.	No evidence of accumulation was observed. Following 14 daily oral administrations of [phenyl-U-^{14}C]-M-01 the major route of elimination was via urine (77 to 82% of dose including cage washes), while faeces contained 16 to 19% of dose. At 144 hours post dose, tissues residues were low (≤ 1.1% of dose). Skin and fur contained the highest residues, with liver, kidneys and adrenals containing the next highest residues. Overall, the multiple dosing did not have any significant impact in the absorption, distribution, metabolism and elimination compared to results after single oral dosing. The same routes of metabolism as seen in the single dose groups were observed in repeat dose animals. The most abundant metabolite detected was a mercapturic acid conjugate of hydroxy-chlorobenzamide (15 to 16% of dose).	[redacted] 2003; M-219491-01-1 KCA 5.1.1/16
PCA single oral low dose EU 87/302/EEC Part B; JMAFF: 59 NohSan 4200; US-EPA OPPTS 870,7485 GLP	[pyridyl-2,6-^{14}C]-AE C657188 (PCA, or M-02) 10 mg/kg bw, 4/sex Single oral gavage dose	Recovery was 94.5% & 92.74% in males & females Radioactivity was detected in the carcass, skin, and fur (0.23% & 0.30% in males & females) The minimum estimated absorption was 86.9% & 87.1% in males & females Parent compound was the major component of urine and faeces (86% & 79% of the administered dose) High oral bioavailability and low potential for accumulation was demonstrated.	[redacted] 2002; M-217250-01-1 KCA 5.1.1/17
Interspecies comparison of in vitro metabolism of [phenyl-UL-^{14}C] fluopicolide using mouse, rat, dog and human liver microsomes Non guideline preliminary study GLP	[Phenyl-U-^{14}C]-Fluopicolide *In vitro* (1 and 10 µM) with liver microsomes from mice, rat, dog and humans	[Phenyl-U-^{14}C]-Fluopicolide was significantly metabolised by liver microsomes from mice, rat, dog, and humans, with a total of 8 metabolites detected. No human-specific metabolites were observed.	[redacted] 2019; M-653630-02-1 KCA 5.1.1/18

Method Guideline GLP compliance	Test Conditions	Result	Reference
Interspecies comparison of in vitro metabolism of [phenyl-UL-^{14}C] fluopicolide using rabbit liver microsomes Non guideline preliminary study GLP	[phenyl-UL-14C]-fluopicolide In vitro (1 and 10 µM) with liver microsomes from New Zealand White rabbits	[Phenyl-U-^{14}C]-Fluopicolide was significantly metabolised by liver microsomes from New Zealand White rabbits, with a total of 10 metabolites detected.	[redacted] 2020; M-685623-01-1 KCA 5.1.1/19
Interspecies comparison of in vitro metabolism of [2,6-Pyridyl-^{14}C]-fluopicolide using rabbit, mouse, rat, dog and human liver microsomes and [phenyl-UL-^{14}C] fluopicolide using rabbit liver microsomes	[Pyridyl-2,6-^{14}C]-AE C638206 *In vitro* (1 and 10 µM) with liver microsomes from mice, rat, dog, and humans	[Phenyl-U-^{14}C]-Fluopicolide was significantly metabolised by liver microsomes from mice, rats, dogs, rabbits, and humans with a total of 16 metabolites detected. No human specific metabolites were observed.	[redacted] 2020; M-689209-01-1 KCA 5.1.1/22
Rat bile excretion study OECD 417 USEPA OPPTS 870.7485 JMAF 12 Nousan No 8147 GLP	[Pyridyl-2,6-14C]-AE C638206 2 mg/kg bw, 6 male bile duct-cannulated rats	Recovery was 105.82% 94.1% of the recovered dose was detected in the bile 4 metabolised were detected in the bile and urine Parent compound was not detected in urine and bile	[redacted] 2020; M-681576-01-1 KCA 5.1.1/20
Rat bile excretion study OECD 417 USEPA OPPTS 870.7485 JMAF 12 Nousan No 8147 GLP	[Phenyl-UL-^{14}C]-AE C638206 2 mg/kg bw, 6 male bile duct-cannulated rats	Recovery was 101.94% 90.1% of the recovered dose was detected in the bile 6 metabolites were identified in the bile and urine Small amounts of parent were detected in the urine	[redacted] 2020; M-681498-01-1 KCA 5.1.1/21

CA 5.1.1 Absorption, distribution, metabolism and excretion by oral exposure

Data Point:	KCA 5.1.1/01
Report Author:	[redacted]
Report Year:	2000
Report Title:	Preliminary toxicokinetic studies in the rat Code: (14C)-AE C638206
Report No:	C008855
Document No:	M-197858-01-1
Guideline(s) followed in study:	EU (=EEC): 94/79/EC; JMAF: NohSan 4200; USEPA (=EPA): OPPTS 870.7485
Deviations from current test guideline:	not specified
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

The absorption, distribution, and excretion of fluopicolide were investigated in bile cannulated rats with radiolabelled fluopicolide ([Phenyl U-14C]-fluopicolide and [2,6-pyridil-14C]-fluopicolide).

Male and female rats (2/sex) received a single oral low dose (25 mg/kg bw) of either [phenyl-U-^{14}C]-fluopicolide or [2,6-pyridyl-^{14}C]-fluopicolide. In addition, one male and one female rat received a single oral high dose (500 mg/kg bw) of [phenyl-U-^{14}C]-fluopicolide. Animals were kept for 7 days following dosing during which time the excreta were collected and quantified and the metabolic profile and identification examined by HPLC and mass spectrometry. After 7 days the animals were sacrificed and the levels of radiolabelled material in the tissues was determined. Blood was collected to determine the pharmacokinetics of fluopicolide.

Absorption following a single oral low dose of [phenyl-U-14C]-fluopicolide or [2,6-pyridyl-14c]-fluopicolide was relatively fast with a Cmax of 1.88 to 2.03 µg/g reached between 6 and 12 hours Tmax) and the calculated terminal half-life ranged from 15.7-32.9 hours in males and females. A Cmax of 29 to 17.69 was reached within 8 hours following administration of a single oral high dose of [phenyl-U-14C]-fluopicolide and the calculated terminal half-life was 39.2 and 32.2 hours in males and females, respectively. The main route of elimination was the faeces with faecal elimination accounting for 79 and 82% in males and females respectively for the low dose [phenyl-U-14C]-fluopicolide groups, 89% (both sexes) for the high dose [phenyl-U-14C]-fluopicolide groups and 76 and 85% in males and females respectively for the low dose [2,6-pyridyl-14c]-fluopicolide groups, with the majority of recovered radioactivity was found in the 0-48h excreta. There were no significant qualitative differences between dose levels or labels regarding the pattern of metabolites; however, a slight sex difference was seen with increased polar excretion in males.

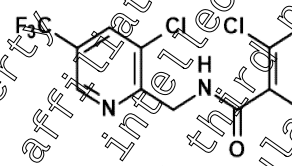
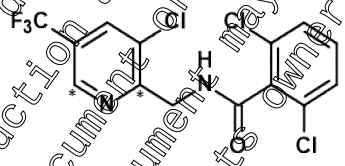
The major metabolite identified in faeces was unchanged parent with metabolites I, II, IV and VII being identified as minor metabolites. The metabolic profile was more complex in urine; minor metabolites identified were I, II, IV, VI, VIII, IX, X, XI, XII, XIV and XV

Three metabolic process were identified comprising aromatic hydroxylation of the benzene ring, glucuronidation of the phase I hydroxyl product and sequential metabolism through the mercapturic acid pathway.

I. Materials and methods

A. Materials

1. Test Material:

IUPAC Name	2,6-dichloro-N-[[3-chloro-5-(trifluoromethyl)-2-pyridinyl]methyl]-benzamide	
CAS Name	Benzamide, 2,6-dichloro-N-[[3-chloro-5-(trifluoromethyl)-2-pyridinyl]methyl]	
Code name	AE C638206	
Common name	Fluopicolide	
Empirical formula	$C_{14}H_8Cl_3F_3N_2O$	
CAS Number	239110-15-7	
Molar mass	383.59 g/mol	
Chemical structure	*Benzene label*	*Pyridine label*
		
	*§Denotes position of label	**Denotes position of label*
Radiolabelled test material	[phenyl-U-^{14}C]-fluopicolide	[2,6-pyridyl-^{14}C]-fluopicolide
Lot number	902AE-1	903AE-1
Radiochemical purity	>99% (HPLC)	>99% (HPLC)
Specific radioactivity	5330 MBq/g (144 µCi/mg)	5880 MBq/g (159 µCi/mg)
Specific radioactivity after dilution with non-radioactive dichloromethane used for the study	Group A: 19.51 µCi/mg Group B: 0.49 µCi/mg Group D: 19.87 µCi/mg Group E: 0.53 µCi/mg	Group C: 20.07 µCi/mg Group F: 19.72 µCi/mg
Stability of test compound	Stable at -20 C	Stable at -20C

2. Vehicle: 1% (w/v) aqueous gum tragacanth

3. Test animals:

Species: Rat
Strain: Sprague Dawley CRL:CD(SD)IGS)BR
Age: 6-8 weeks
Weight at dosing: 163-214g
Source: [redacted]
Acclimation period: Yes
Identification: Ear markings
Diet: Free access to food (pelleted laboratory rodent diet, Harlan Tekland 9607 TRM SQC), withheld for 18 hours prior to dosing
Water: Provided *ad libitum* (Mains supply from bottles attached to cages)
Housing: Housed in single sex groups of 3-4 animals in plastic holding cages with woodchip bedding

Environmental conditions

Temperature:	21 ± 2˚C
Humidity:	Not stated
Photoperiod:	12 hours

B. Study design and methods

1. In life dates: July 1999 to September 1999

2. Dose regimen and design of tests

Table 5.1.1- 1 :Study design

Group no.	Radiolabelled test substance	Nominal dose	Actual mean doses (mg/kg bw)	Conc. (mg/g)	Rats/sex	Collection of samples during the test and at sacrifice	Duration
Elimination/tissue residue study							
A	[phenyl-U-^{14}C]-fluopicolide	Low dose: 25 mg/kg bw	Males: 24.43 Females: 25.65	4.931	2/sex	Urine, faeces, cage wash, expired air*, blood, plasma, organs, tissues	7 days
B	[phenyl-U-^{14}C]-fluopicolide	High dose: 500 mg/kg bw	Male: 487.66 Female: 495.04	49.504	1/sex	Urine, faeces, cage wash, expired air*, blood, plasma, organs, tissues	7 days
C	[2,6-pyridyl-^{14}C]-fluopicolide	Low dose: 25 mg/kg bw	Males: 22.71 Females: 22.44	4.558	2/sex**	Urine, faeces, cage wash, expired air*, blood, plasma, organs, tissues	7 days
Blood level study							
D	[phenyl-U-^{14}C]-fluopicolide	Low dose: 25 mg/kg bw	Males: 24.66 Females: 24.40	4.996	2/sex	Blood	96 hours
E	[phenyl-U-^{14}C]-fluopicolide	High dose: 500 mg/kg bw	Males: 487 Females: 510.97	50.220	2/sex	Blood	96 hours
F	[2,6-pyridyl-^{14}C]-fluopicolide	Low dose: 25 mg/kg bw	Males: Females:	5	2/sex	Blood	96 hours

* Assayed on days 1 and 2 only, contained <1% administered dose so not used for the remainder of the study
**One female died after 48h therefore the data are not included

3. Dosing

The doses were prepared by mixing adequately weighed amounts of radiolabelled and non-radiolabelled fluopicolide in dichloromethane solvent. The solvent was removed by desiccation and the co-precipitated compound was resuspended in 1% (w/v) aqueous gum tragacanth. The dose suspensions were then milled with glass beads for approximately 20-45 minutes to achieve homogeneity. The doses (as detailed in table **5.1.1- 1** above) were administered to each rat *via* a single oral gavage dose.

4. Collection of excreta

After administration of the radiolabelled test substance, animals in groups A, B and C (elimination study) were kept individually in glass metabolism cages which allowed for separate and quantitative collection of urine and faeces. Urine and faeces were collected separately at 6 hours (urine only) and 24 hours following dosing, and thereafter on days 2, 3, 4, 5, 6 and 7.

Cage washing was conducted by rinsing with water/ethanol after the final sample collection and the cage washings were kept separately for analysis.

Expired air was passed through carbon dioxide traps containing 2-methoxyethanol/ethanolamine (4:1 v/v). These traps were assayed on Days 1 and 2 and were found to contain less than 1% of the administered radioactivity and were therefore not used for the remainder of the study.

5. Blood and plasma

A sample of blood was taken at sacrifice from the animals of groups A, B and C (elimination study) for analysis and the remainder centrifuged to yield plasma.

In the blood level study (groups C, D and E), approximately 100 µL of blood was collected from a lateral tail vein into a capillary tube and expelled onto an adsorbent pad (Canberra Packard Combustopads/Combustocones) for weighing and assaying at the following time-points: 0.5, 1, 2, 4, 6, 8, 12, 24, 30, 48, 72 and 96 hours after dosing.

6. Sacrifice

Elimination study: Animals were killed by aortic exsanguination under deep isoflurane anaesthesia, at 7 days following dose administration.

Blood level study: Animals were killed by cervical dislocation following the final blood collection (96 hours following dosing).

7. Tissues and organs collected at sacrifice

In groups A, B and C (elimination study) the following organs and tissues were collected at sacrifice: brain, kidneys, liver, ovaries, testes (including prostate and epididymis), renal fat and residual carcass (containing gastrointestinal tract). Unless processed immediately, the samples were frozen until required.

8. Measurement of radioactivity

Radioactivity was measured by liquid scintillation counting with automatic external standard quench correction. Corrected dpm of less than background cpm were considered to be below the limit of quantification (<LOQ).

9. Toxicokinetic analysis and data processing

In this study, the software TOPFIT (version 2.0) was used to calculate the pharmacokinetic parameters.

Weights (pots, tissues, homogenates, aliquots) were either captured directly from balances by the DEBRA database system, recorded manually onto raw data sheets and then edited onto the system or manually entered via a keyboard directly onto the DEBRA 5.1 a database running on a Novell network of computers. Scintillation counts were stored on magnetic disk and subsequently transferred to the system. Printouts from balances and scintillation counters were retained as raw data.

Weights (pots, extracts, aliquots, residues) generated during extraction were either captured directly from balances by the TRACE database system, recorded manually onto raw data sheets and then edited onto the system or manually entered via a keyboard directly onto the TRACE 1.1a database running on a Novell network. Scintillation counts were stored on magnetic disk and subsequently transferred to the system. Printouts from balances and scintillation counters were retained as raw data.

The concentration of tissue residue in all samples was calculated. Means and standard deviations for each analysis and for individual values in each group were also calculated. All calculations were carried out automatically by the DEBRA system.

10. Preparation of samples for quantification of radioactivity

Samples of all excreta, cage washes and tissues were prepared for analysis in triplicate where possible. Solubilisation was facilitated by incubation at approximately 40°C. The following methods were used:

i) Urine, cage wash and plasma: Weighed aliquots (0.025-1.0mL) were added directly to Starscint scintillant for liquid scintillation counting (LSC).

ii) CO_2 traps: $^{14}CO_2$ was trapped in approximately 200mL of a mixture of ethanolamine: 2-methoxyethanol (1:4 v/v). Weighed aliquots (2.0mL) of the mixture were added directly to Starscint for LSC.

iii) Blood: Weighed aliquots of blood (0.10-0.25mL) were combusted in a sample oxidiser and the $^{14}CO_2$ trapped in Carbosorb/Permafluor E+ (Canberra-Packard) for measurement of radioactivity by LSC.

iv) Ovaries and fat: Whole organs or weighed aliquots were taken and solubilized in SHT (1.0 mL) and water (1.0 mL) for up to 48 hours, acidified with glacial acetic acid (0.5ml) and mixed with Starscint scintillant for LSC.

v) Gastro-intestinal tract and carcass: Digested in sodium hydroxide (10 M, 40% w/v) at approximately 40°C for 15 days. After homogenisation aliquots of known mass (0.5g) were sampled, acidified with glacial acetic acid (1.0 mL) and mixed with Starscint scintillant for LSC.

vi) All other tissues: The total sample was homogenised in water and weighed aliquots (1.0ml) were taken, solubilised and prepared for scintillation counting as in (iv) above, without the additional water.

vii) Faeces: Water was added to faeces to give an approximate concentration of 1:10 (w/v) (faeces : water) after which the sample was homogenised. Weighed aliquots (0.5ml) of homogenate were taken, SHT (1.0mL) was added and the mixture was allowed to digest for 24-48 hours, acidified with glacial acetic acid (0.5mL) and mixed with Starscint scintillant for LSC.

11. Preparation of samples for determination of metabolic profiles

Metabolic profiles were determined in pooled urine samples (6 and 24 hour) and pooled 24-hour faeces samples in both sexes at all dose levels.

The pooled faecal homogenates were centrifuged at 3000 rpm for 10 minutes, the aqueous supernatants were then decanted and quantified. The residual material was subsequently extracted with acetonitrile (approximately 100-200 mL) and stirred for over one hour on a magnetic stirrer at room temperature. After centrifugation at 3000rpm for 10 minutes the aqueous supernatants were decanted and quantified.

12. High performance liquid chromatography (HPLC)

High performance liquid chromatography was used for the analysis of radiochemical purity as well as for analysis of urine and faeces samples. HPLC analysis was performed using the following equipment:

Pump	Jasco PU-980 or Merck Hitachi L-6200
Gradient maker	Jasco LG-980-02
UV detector	Jasco UV 975 or Merck Hitachi L-4000
Radio detector	Reeve analytical 9701 fitted with either a heterogeneous flow cell (200 µl) or a homogeneous flow cell (1.0 ml) and series 27000 data system
Homogeneous mixer	Reeve analytical 9802
Column	Columbus C_{18} 5µM, 250 x 4.6mm

The separation was carried out on a reversed phase column using an acidic water/acetonitrile gradient. The flow rate was 1 ml/min and the UV wavelength 254nm; this system successfully separated fluopicolide from the metabolite 2,6-dichlorobenzamide (BAM) and moved BAM away from the solvent front.

13. Identification of metabolites

Following examination of the HPLC radio traces, the following 24h samples were examined by mass spectrometry to identify the metabolites present:

24h male urine - low dose [phenyl-U-14C]-fluopicolide label

24h female urine - low dose [phenyl-U-14C]-fluopicolide label

24h male urine - low dose [2,6-pyridyl-14c]-fluopicolide label

24h female urine extract - low dose [2,6-pyridyl-14c]-fluopicolide label

24h male faecal water extract - low dose [phenyl-U-14C]-fluopicolide label

24h female faecal acetonitrile extract - low dose [phenyl-U-14C]-fluopicolide label.

II. Results and Discussion

A. Recovery

The mean overall recovery of radioactivity for groups A, B and C (elimination study) from measurements in urine, faeces and tissues, in addition to carcass and GIT at sacrifice was approximately 87.7% and 95.2% the administered radioactivity in males and females respectively for group A, 95.6% and 94.5% in males and females respectively for group B and 85.5% and 93.4% in males and females respectively for group C. A summary of the radioactivity in percent of the administered dose found in excreta and in organs and tissues at sacrifice is presented in table 5.1.1 below.

Table 5.1.1- 2 Recovery of radioactivity following single oral administration of [^{14}C]-fluopicolide, data presented as % of dose administered

Group	A		B		C	
Dose	25 mg/kg bw		500 mg/kg bw		25mg/kg bw	
Experiment	single low dose [phenyl-U-^{14}C]-fluopicolide		single high dose [phenyl-U-^{14}C]-fluopicolide		single low dose [2,6-pyridyl-^{14}c]-fluopicolide	
Duration, No./sex	168 h, 2/sex		168 h, 1/sex		168 h, 2/sex*	
	% administered dose		% administered dose		% administered dose	
	Male	Female	Male	Female	Male	Female
Urine (0-168 h)	8.123	12.333	5.584	5.076	9.777	8.433
Faeces (0-168 h)	79.028	82.437	88.810	89.351	75.571	84.762
Cage wash (168 h)	0.173	0.088	0.063	0.031	0.061	0.116
CO2 (0-48 h)	0.047	0.063	0.021	0.021	0.016	0.007
Carcass/GIT	0.251	0.223	0.052	0.049	0.031	0.036
Plasma	0.000	0.000	0.000	0.000	0.000	0.000
Blood	0.002	0.004	0.001	0.002	0.001	0.001
Brain	0.002	0.001	0.000	0.000	0.000	0.000
Renal fat	0.000	0.000	0.000	0.000	0.000	0.000
Liver	0.102	0.075	0.024	00024	0.032	0.023
Kidney	0.011	0.010	0.002	0.002	0.002	0.002
Ovaries	-	0.000	-	0.000	-	0.000
Testes	0.002	-	0.000	-	0.000	-
Total	87.740	95.236	95.554	94.508	85.490	93.379

*one female died after 48-hours owing to an air pump failure, therefore these results are excluded, **including epididymis and prostate gland

B. Absorption

Following administration of the low dose the compound was absorbed relatively rapidly, with similar blood profiles seen in males and females dosed with either the benzene or pyridine label. The time to maximum concentration was 12 and 8 hours respectively in the males and females dosed with the benzene label and 6 hours in both the males and females dosed with the pyridine label.

The C_{max} (maximum measured concentration of blood) was slightly lower in the females dosed with the benzene label at 0.95 µg/g, than seen in males and females dosed with the pyridine label (1.76 and 2.03 µg/g respectively) and in males dosed with the benzene label (1.88µg/g).
Calculated half-lives ranged from 15.7 to 32.9 hours in the blood of males and females of the benzene label dosed animals respectively, to between 32.3 and 39.2 hours in the blood of females dosed with the pyridine label respectively.
The clearance (g/min) was 10.2 and 11.5 in males and females administered the benzene label and 9.70 and 9.06 in males and females administered the pyridine label.

Table 5.1.1- 3: Time course of radioactivity in the blood of the rats following a single oral administration of [^{14}C]-fluopicolide, expressed as dose equivalent concentration in µg/g

Group	D		E		F	
Dose	25 mg/kg	25 mg/kg benzene label	500 mg/kg benzene label	500 mg/kg benzene label	25 mg/kg pyridine label	25 mg/kg pyridine label
Experiment	single low dose [phenyl-U-^{14}C]-fluopicolide	single low dose [phenyl-U-^{14}C]-fluopicolide	single high dose [phenyl-U-^{14}C]-fluopicolide	single high dose [phenyl-U-^{14}C]-fluopicolide	single low dose [2,6-pyridyl-^{14}c]-fluopicolide	single low dose [2,6-pyridyl-^{14}c]-fluopicolide
No./sex	2 males	2 females	2 males	2 females	2 males	2 females
Time (post administration)	[µg a.s. equiv./g]$_{plasma}$					
0.5	0.342	0.196	2.395	2.267	0.436	0.635
1	0.685	0.461	3.930	2.887	0.671	0.842
2	0.907	0.702	6.374	4.817	0.734	0.929
4	1.036	0.842	9.601	6.261	1.323	1.227
6	1.019	0.888	8.791	17.688	1.759	2.031
8	1.071	0.946	29.003	13.964	1.731	1.857
12	1.875	0.755	23.326	13.461	1.248	0.857
24	0.723	0.344	23.011	17.085	0.299	0.360
30	0.348	0.273	12.776	12.199	0.264	0.355
48	0.127	0.224	3.286	4.841	0.167	0.232
72	0.078	0.176	1.630	3.277	0.130	0.199
96	NA	NA	NA	NA	NA	NA

C. Distribution

The levels of test item in the different compartments and tissues was measured in groups A, B and C (see table 5.1.1-4 below).

In group A (low dose [phenyl-U-14C]-fluopicolide label), the levels of radioactivity in the individual tissues at necropsy, including the carcass and GI tract, accounted for less than 0.5% of the dose administered, and residues were below 0.10 µg/g in all tissues, with the exception of liver (0.429/0.388 µg/g in males/females), kidney (0.217/0.258 µg/g in males/females) and blood (0.234 µg/g in females).

In group B (high dose [phenyl-U-14C]-fluopicolide label), the levels of radioactivity in all tissues (including carcass and GI tract) accounted for less than 0.1% of the administered dose. Residues were generally around or below 0.200 µg/g in all tissues, with the exception of liver (2.163/2.019 µg/g in males/females), kidney (0.961/1.201 µg/g in males/females) and blood (1.093/1.531 µg/g in males/females).

In group C (low dose [2,6-pyridyl-14c]-fluopicolide label), the levels of radioactivity recovered in the tissues, carcass and GI tract accounted for less than 0.1% of the administered dose. Residues were at or below 0.100 µg/g in all tissues, with the exception of the liver in males (0.126 µg/g).

Table 5.1.1- 4: Radioactive residues in organs and tissues at sacrifice at 168 hours expressed as % administered dose and µg equivalents fluopicolide/g tissue

Group	A		B		C	
Dose, route	25 mg/kg bw		500 mg/kg bw		25mg/kg bw	
Experiment	single low dose [phenyl-U-^{14}C]-fluopicolide		single high dose [phenyl-U-^{14}C]-fluopicolide		single low dose [2,6-pyridyl-^{14}c]-fluopicolide	
Duration, No./sex	168 h, 2/sex		168 h, 1/sex		168 h, 2/sex*	
	% administered dose		% administered dose		% administered dose	
	Male	Female	Male	Female	Male	Female
Carcass/GIT	0.251	0.225	0.092	0.049	0.031	0.036
Plasma	0.000	0.000	0.000	0.000	0.000	0.000
Blood	0.002	0.004	0.001	0.002	0.001	0.001
Brain	0.002	0.001	0.000	0.000	n.d	0.000
Renal fat	0.000	0.000	0.000	0.000	n.d	0.000
Liver	0.102	0.075	0.024	0.024	0.032	0.023
Kidney	0.011	0.010	0.002	0.002	0.002	0.002
Ovaries	-	0.000	-	0.000	-	0.000
Testes**	0.002	-	0.000	-	0.000	-
Total	0.371	0.316	0.080	0.029	0.065	0.063
	µg equivalents fluopicolide/g tissue		µg equivalents fluopicolide/g tissue		µg equivalents fluopicolide/g tissue	
	Male	Female	Male	Female	Male	Female
Carcass/GIT	0.049	0.55	0.218	0.220	0.006	0.008
Plasma	0.011	0.011	0.067	0.073	0.004	0.004
Blood	0.095	0.234	1.093	1.531	0.057	0.100
Brain	0.047	0.028	0.119	0.197	0.002	0.002
Renal fat	0.018	0.027	0.115	0.180	0.005	0.006
Liver	0.429	0.388	2.165	2.019	0.126	0.097
Kidney	0.217	0.258	0.961	1.261	0.035	0.057
Ovaries	-	0.030	-	0.180	-	0.007
Testes*	0.025	-	0.101	-	0.002	-

*one female died after 48-hours owing to an air pump failure, therefore these results are excluded, **including epididymis and prostate gland

The distribution of the test item was followed by measuring the concentration of the total radioactivity in the plasma of groups D, E and F (see table 5.1.1-5 below).

For the low dose [phenyl-U-14C]-fluopicolide label, the plasma radioactivity (C_{max}) peaked at 1.88 µg/g and 0.95 µg/g in males and females respectively and was reached at a respective 12 and 8 hours post administration. This was followed by an elimination phase ($t_{1/2}$) of 15.7 hours in males and 32.9 hours in females. The area under the curve (AUC) was 38.6 Mg*hour/kg in males and 26.98 Mg*hour/kg in females.

For the low dose [2,6-pyridyl-14c]-fluopicolide label the plasma radioactivity peaked at 1.76 µg/g and 2.03 µg/g, 6 hours post dose in male sand females respectively, followed by an elimination phase ($t_{1/2}$) of 39.2 hours in males and 32.2 in females. The area under the curve was 33.98 Mg*hour/kg in males and 35.89 in females.

Table 5.1.1- 5: Pharmacokinetic parameters of [^{14}C]-fluopicolide, following a single oral administration

Parameter	Single low dose [phenyl-U-14C]-fluopicolide	Single low dose [phenyl-U-14C]-fluopicolide	Single low dose [2,6-pyridyl-14c]-fluopicolide	Single low dose [2,6-pyridyl-14c]-fluopicolide
Dose mg/kg bw	24.66	24.40	24.12	24.63
Duration h	96	96	96	96
Sex of rats	Male	Female	Male	Female
No. of animals	2	2	2	2
Mean rat weight g	200.5	185.25	207.5	193.65
C_{max} µg/g	1.88	0.95	1.76	2.03
t_{max} Time (h)	12	8	6	6
$t_{1/2}$ h	15.7	32.9	39.2	32.2
AUC Mg*hour/kg	38.60	26.98	33.98	35.89
Clearance g/min	10.2	11.5	9.70	9.06

D. Excretion

In all groups (A, B and C), faeces appeared to be the major route of elimination, accounting for approximately 80% of the administered dose in group A (low dose [phenyl-U-14C]-fluopicolide label). Similarly, in group B (high dose [phenyl-U-14C]-fluopicolide label) 63-77% of the administered dose was recovered in the faeces in the first 24-hours following dosing and in group C (low dose [2,6-pyridyl-14c]-fluopicolide label). 65-79% of the administered dose was recovered in the faeces, also within 24-hours post-dose.

Urine was a minor route of elimination in all dose groups, accounting for 8-12%, 5% and 8-9% of the administered dose in groups A, B and C, respectively.

In all cases, negligible amounts of radioactivity were recovered as ^{14}-C labelled carbon dioxide within the first 24-hours, indicating the metabolic stability of the test compound (see table 5.1.1-2).

Table 5.1.1- 6: Recovery of radioactivity in excreta, data presented as % of dose administered

Group	A				B		C		
Dose, route	**25 mg/kg bw**				**500 mg/kg bw**		**25mg/kg bw**		
Experiment	**single low dose [phenyl-U-^{14}C]-fluopicolide**				**single high dose [phenyl-U-^{14}C]-fluopicolide**		**single low dose [2,6-pyridyl-^{14}c]-fluopicolide**		
Duration, No./sex	**168 h, 2/sex**				**168 h, 1/sex**		**168 h, 2/sex***		
	% administered dose				**% administered dose**		**% administered dose**		
	Male		**Female**		**Male**	**Female**	**Male**		**Female**
	Mean	SD	Mean	SD	Mean	Mean	Mean	SD	Mean
Urine	8.123	0.181	12.333	2.452	5.584	5.076	9.777	2.900	8.433
Faeces	79.028	2.502	82.437	0.146	88.810	89.351	75.571	9.971	84.762
Cage Wash	0.173	0.000	0.088	0.002	0.063	0.031	0.061	0.016	0.116
Total	87.324	2.683	94.858	2.600	94.457	94.458	85.409	12.887	93.311

*one female died after 48-hours owing to an air pump failure, therefore these results are excluded

E. Metabolism

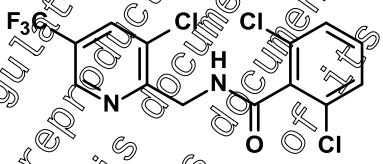
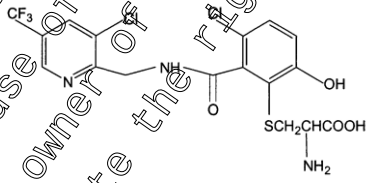
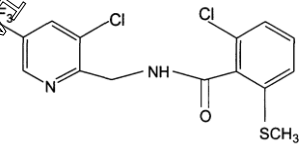
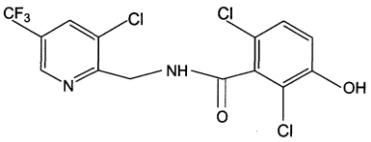
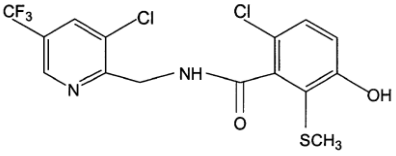
Fluopicolide was extensively metabolised with no parent compound being present in the majority of the recovered radioactivity being excreted close to the solvent front.

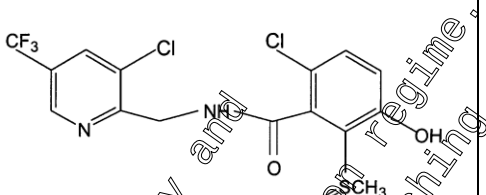
Faeces

The majority of the dose was recovered in the faeces. HPLC analysis of the faecal water and acetonitrile extracts showed that AE C638206 was the major component in faeces along with 2 unknown metabolites in the aqueous extracts. This was confirmed with mass spectrometry, in which the major metabolite in the 0-24-hour faeces sample was AE C638206 at both dose levels. Other metabolites (at lower concentrations) were also identified as metabolites I, II, IV, V and VII.

The metabolites identified in faeces are shown in the table below:

Table 5.1.1- 7 Metabolites identified in the faeces following single oral high and low doses of [phenyl-U-^{14}C]-fluopicolide and a single oral low dose of [2,6-pyridyl-^{14}c]-fluopicolide

Metabolite code	Description	Chemical structure
Fluopicolide (AE C638206, BCS-AM59797)	Unchanged parent was the major metabolite identified in 0-24 h faeces at both dose levels	
Metabolite I	Hydroxylation and replacement of a chlorine atom on the benzene ring with a cysteine molecule.	
Metabolite II	Corresponds to compound containing an s-methyl group but without hydroxyl function.	
Metabolite IV	The insertion of molecular oxygen via hydroxylation on the benzene ring	
Metabolite V	Further metabolism of the cysteine side chain of metabolite I to form the s-methyl group	

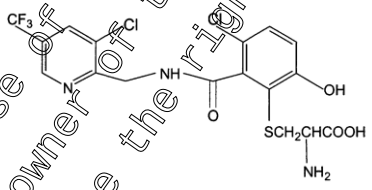
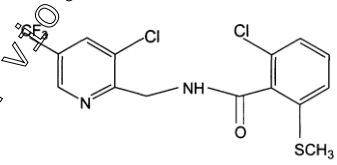
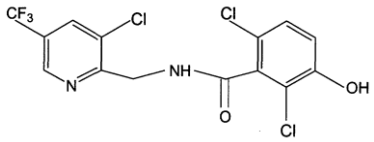
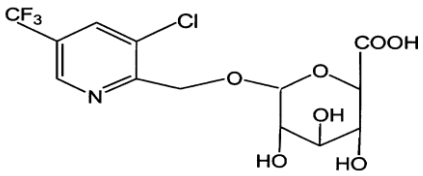
Metabolite code	Description	Chemical structure
Metabolite VII	Oxidation of the s-methyl group of metabolite V.	

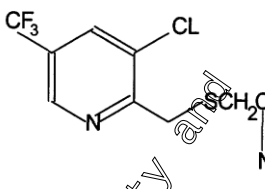
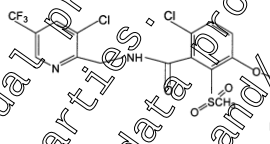
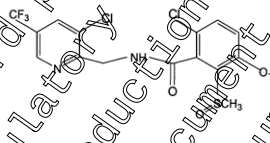
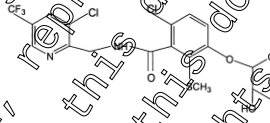
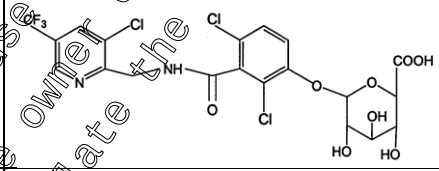
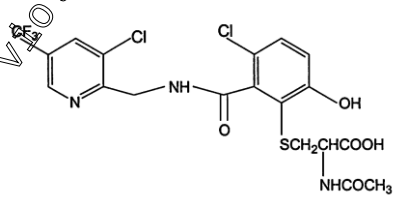
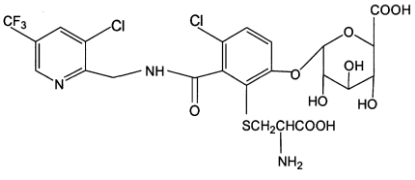
Urine

Initial HPLC analysis of the urine samples showed that AE C638206 underwent extensive metabolism. There were no significant differences between the high and low dose, therefore only the low dose samples (which contained the greatest amount of radioactivity) were used for identification purposes. Owing to the large number of metabolites present it was not possible to identify all of the metabolites, however 11 metabolites were identified in the urine.

The metabolites identified in urine are shown in the table below.

Table 5.1.1- 8: Metabolites identified in the urine following single oral low doses of [phenyl-U-^{14}C]-fluopicolide and [2,6-pyridyl-^{14}C]-fluopicolide

Metabolite code	Description	Chemical structure
Metabolite I	Hydroxilation and replacement of a chlorine atom on the benzene ring with a cysteine molecule.	
Metabolite II	Corresponds to compound containing an s-methyl group but without hydroxyl function.	
Metabolite IV	The insertion of molecular oxygen via hydroxylation on the benzene ring	
Metabolite VI	Cleavage of the amide group formation of a hydroxyl group conjugated to a glucuronic acid on the pyridine side.	

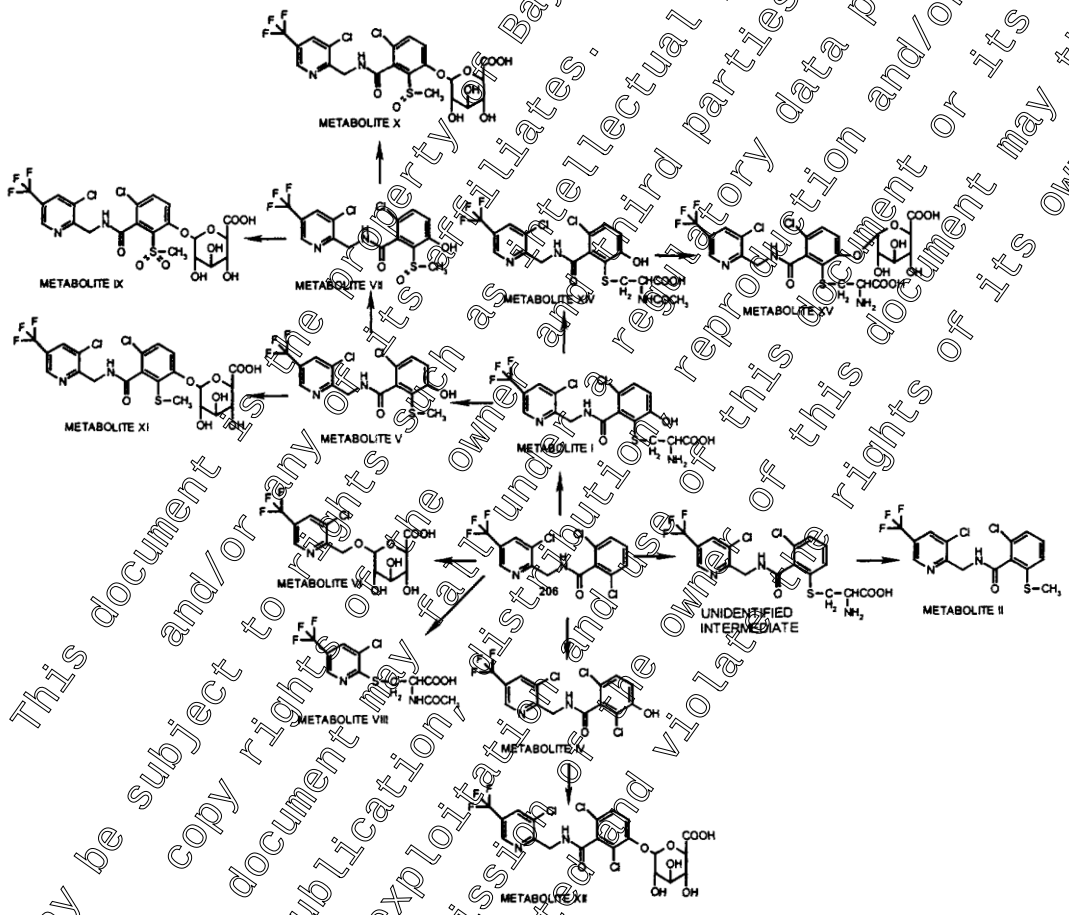
Metabolite code	Description	Chemical structure
Metabolite VIII	Replacement of the pyridine side chain with a mercapturic acid (identity in doubt)	
Metabolite IX	Further oxidation of the sulphoxide group of metabolite VII to form a sulphone, combined with glucurionidation of the aromatic hydroxyl function.	
Metabolite X	Gluocuronide conjugate via glucuronidation of metabolite VII	
Metabolite XI	Gluocuronide conjugate via glucuronidation of metabolite V	
Metabolite XII	Gluocuronide conjugate via glucuronidation of metabolite IV	
Metabolite XIV	Mercapturic acid of AE C638206 formed through metabolism of the cysteine group of metabolite I	
Metabolite XV	Gluocuronide conjugate via glucuronidation of metabolite XIV	

The metabolic profile indicated three major reactions in the metabolism of fluopicolide:

- Aromatic hydroxylation of the benzene ring
- Glucuronidation of the phase 1 hydroxy product
- Sequential metabolism through the mercapturic acid pathway

The proposed metabolic pathway of fluopicolide is presented below:

Figure 5.1.1-1: Proposed metabolic pathway



III. Conclusions

- Absorption following a single oral low dose of [phenyl-U-14C]-fluopicolide or [2,6-pyridyl-14c]-fluopicolide was relatively fast with a Cmax of 1.88 to 2.03 µg/g reached between 6 and 12 hours Tmax) and the calculated terminal half-life ranged from 15.7-32.9 hours in males and females. A Cmax of 29 to 17.69 was reached within 8 hours following administration of a single oral high dose of [phenyl-U-14C]-fluopicolide and the calculated terminal half-life was 39.2 and 32.2 hours in males and females, respectively.
- Fluopicolide was poorly absorbed and rapidly excreted in the faeces of male and female rats following a single oral high or low dose.
- The main route of elimination was the faeces with faecal elimination accounting for 79 and 82% in males and females respectively for the low dose [phenyl-U-14C]-fluopicolide groups, 89% (both sexes) for the high dose [phenyl-U-14C]-fluopicolide groups and 76 and 85% in males and females respectively for the low dose [2,6-pyridyl-14c]-fluopicolide groups.
- The majority of recovered radioactivity was found in the 0-48h excreta. 97 and 98% was found in males and females for both doses of [phenyl-U-14C]-fluopicolide, whilst 97-99% was found in males and females administered low dose [2,6-pyridyl-14c]-fluopicolide.
- There were no significant qualitative differences between dose levels or labels regarding the pattern of metabolites; however, a slight sex difference was seen with increased polar excretion in males.
- The major metabolite identified in faeces was unchanged parent with metabolites I, II, IV and VII being identified as minor metabolites.
- The metabolic profile was more complex in urine; minor metabolites identified were I, II, IV, VI, VIII, IX, X, XI, XII, XIV and XV.
- Three metabolic process were identified comprising aromatic hydroxylation of the benzene ring, glucuronidation of the phase I hydroxyl product and sequential metabolism through the mercapturic acid pathway.

Assessment and conclusion by applicant:

The study is valid and acceptable to give an indication of the route and rate of absorption, distribution and elimination of fluopicolide in the rat and to compare the metabolic profiles in urine and faeces.

Data Point:	KCA 5.1.1/02
Report Author:	[redacted]
Report Year:	2001
Report Title:	[phenyl-U-14C] - AE C638206 Single high & low dose rat A.D.E. study
Report No:	C017703
Document No:	M-204781-01-1
Guideline(s) followed in study:	EU (=EEC): 87/302, part B; JMAF: 59 Nohsan 4200; USEPA (=EPA): OPPTS 870.7485
Deviations from current test guideline:	not specified
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

The absorption, distribution, and excretion of fluopicolide were investigated with radiolabelled fluopicolide ([Phenyl U-14C]-fluopicolide).

A single oral gavage dose of [Phenyl U-14C]-fluopicolide was administered to male and female Sprague Dawley rats at a nominal dose of 10 mg/kg bw (Single Oral Low Dose) or 100 mg/kg bw (Single Oral High Dose). Urine and faeces were collected at regular intervals from dosing until sacrifice at 168-hours and the radioactivity in the samples determined by liquid scintillation counting (LSC). At sacrifice, the organs, tissues, blood and carcass were collected, and the radioactivity measured by LSC.

Recovery was high, with 96.38 ± 5.80% of the administered dose being recovered in the excreta along with remaining tissues and carcass at sacrifice.

Excretion was fast and almost complete at 48-hours post-dose. The mean level of faecal elimination in rats from the low dose group was 82.58% and 82.09% of the administered dose for males and females respectively and 87.46% and 88.28% in males and females respectively of the high dose group. The corresponding mean recoveries of radioactivity in the urine for male and female rats respectively were 10.30% and 13.09% in the low dose group and 5.39% and 6.58% in the high dose group.

The amount of radioactivity remaining in the total body at sacrifice (168 hours following dosing) was low (approximately 1%) and relatively similar between sexes and doses. The greatest amounts of radioactivity were found in the liver and kidneys of males and females of both dose groups, and additionally in the skin and fur of high dose females. Radioactivity in the thyroids of both sexes of the low and high dose groups and in the bone & marrow of males and females of the high dose group was below the limit of detection (twice background level).

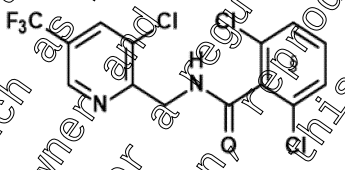
In the Low Dose Group, the liver and kidneys displayed the highest tissue/plasma ratios reaching 24.55 and 18.09 respectively for the males and 22.37 and 18.58 for the females. For both dose groups, however, the high blood/plasma ratio indicated that the test substance was present in greater quantities in the blood cells than in the plasma therefore the tissue/blood ratio more accurately reflects the equilibrium between blood and tissue compartments. By using these measurements, in the low dose group only the liver (3.62), kidneys (2.64), harder's gland (1.28), heart (1.26) and adrenals (1.17) gave a ratio above one in males and liver (1.68) and kidneys (1.38) in females. The same tissues gave a ratio above one in males and females of the high dose group, with liver showing the highest ratio in males (3.21) and females (2.97). These results would therefore suggest that no accumulation has occurred in tissues.

Based on renal excretion, the oral absorption of [phenyl-U-14C]-fluopicolide can be estimated at 11.55% and 14.08% for the males and females from the Low Dose Group respectively, and 6.14% and 7.61% for the males and females from the High Dose Group respectively. However, owing to the large amount of faecal excretion it is likely that a large proportion of the dose was absorbed and excreted via the bile, thus making the actual oral absorption value much higher.

I. Materials and methods

A. Materials

1. Test Material:

IUPAC Name	2,6-dichloro-N-[[3-chloro-5-(trifluoromethyl)-2-pyridinyl]methyl]-benzamide
CAS Name	benzamide, 2,6-dichloro-N-[[3-chloro-5-(trifluoromethyl)-2-pyridinyl]methyl]
Code name	AE C638206
Common name	Fluopicolide
Empirical formula	$C_{14}H_8Cl_3F_3N_2O$
CAS Number	239110-15-7
Molar mass	383.59 g/mol
Chemical structure	 □ *denotes position of label*
Radiolabelled test material	[phenyl-U-^{14}C]-fluopicolide*
Lot number	901CU-2
Radiochemical purity	> 99% (by HPLC)
Specific radioactivity	59.5 mCi/mmole or 2.202 GBq/mmole
Specific radioactivity of dose suspensions	Checked by HPLC and radio assay
Stability of test compound	Stable at -20˚C

*referred to as [phenyl-U-14C]-AE C638206 in the report

2. Vehicle: Aqueous methyl cellulose (0.75% w/w)

3. Test animals:

Species:	Rat
Strain:	Sprague Dawley, CD
Age:	Not stated
Weight at dosing:	151-175g
Source:	[redacted]
Acclimation period:	7 days
Identification:	Ear tattoo
Diet:	Certified rodent diet M20 (Pietrement, Provins, France)
Water:	Provided *ad libitum* (filtered, softened water from municipal supply)
Housing:	Housed in metal wire-mesh bottomed cages in metal trays containing absorbent paper during acclimatisation and in specialised metabolism cages thereafter

Environmental conditions

Temperature:	22 ± 2˚C
Humidity:	55 ± 15%
Photoperiod:	12 hours
Air changes	Average 15 changes/hour

4. Preparation of dosing solutions

The doses were prepared by mixing weighed amounts of radiolabelled and non-radiolabelled material in acetonitrile to achieve a homogenous solution at the desired specific radioactivity. The solution was then gently evaporated under a stream of nitrogen, appropriate weights of aqueous methyl cellulose (0.75% w/w) added and the resulting solution ground to produce a suspension. The dose suspension was of sufficient quantity and concentration to provide a dose of 1g per 200 g rat body weight. The dose suspensions for the low and high dose groups were prepared on the day of dosing and assayed by HPLC for active ingredient content and for radioactivity content (by LSC) before, during and following the dosing procedure.

B. Study design and methods

1. In life dates: September 12, 2000 to April 19, 2001

2. Dose regimen and design of tests

Table 5.1.1- 9: Study design

Administered single dose of [phenyl-U-^{14}C]-fluopicolide , route (experiment)	Number of rats and sex	Collection of samples during the test and at sacrifice	Duration
10 mg/kg bw, oral (single low dose)	4 males 4 females	Urine, faeces, cage wash, , blood, plasma, organs, tissues, carcass	7 days
100 mg/kg bw, oral (single low dose)	4 males 4 females	Urine, faeces, cage wash, , blood, plasma, organs, tissues, carcass	7 days

The rats each received a single dose of [phenyl-U-14C]-fluopicolide at a target dose of either 10 mg/kg bw/d (low dose) or 100 mg/kg bw/d. Urine and faeces samples were collected at intervals until sacrifice at 168-hours, when the blood, organs, tissues and carcass were collected.

3. Dosing

Adequate volumes of the suspension of the test compound in aqueous methyl cellulose (0.75% w/w) were administered to fasted (18-hours) rats *via* a single oral gavage dose. The high and low doses for each individual animal were calculated based on the weights of the animal prior to dosing (food was replaced one-hour post dose and remained available *ad libitum* throughout the remainder of the study).

4. Collection of excreta

Following administration of the radiolabelled test substance, animals of the high and low dose groups were kept in specialized individual metabolism units (Jencon's metabowls MK III), which permitted the separate quantitative collection of urine and faeces, for a maximum of 168-hours following dosing. Both urine and faeces were collected frozen over solid CO_2 in weighed polystyrene boxes; cardeal cotton protected the samples from light.

Urine was collected at 0-6 and 6-24 hours and subsequently at 24-hour intervals following dosing whilst faeces was collected at 24-hour intervals following dosing. Cages were washed with distilled water for each 24-hour period post-dose and then with acetonitrile at the end of the in-life phase; cage washes were retained for analysis. The radioactivity content was determined by HPLC.

5. Blood and plasma

Cardiac blood samples were taken from each animal at sacrifice, and plasma prepared by centrifugation (approximately 2000 x g for 10 minutes).

6. Sacrifice

All animals were killed by exsanguination under Imogene 500 anesthesia at 7 days following dose administration.

7. Tissues and organs collected at sacrifice

The following organs and tissues were collected at sacrifice: Liver, kidney, heart, lungs, brain, spleen, pancreas, fat (abdominal), skeletal muscle, eyes, ovaries, testes, intestinal tract plus contents, stomach plus contents, bone (femur) and marrow, adrenals, uterus, thyroid, harderian glands, skin and fur. The residual carcass was retained for analysis. Samples were processed immediately if possible or were frozen until required.

8. Measurement of radioactivity

Radioactivity was measured by liquid scintillation counting with automatic external standard quench correction. The limit of detection was taken to be twice the background values for blank samples in appropriate scintillation cocktails.

9. Data processing

The calculations of % dose recovery and compound concentrations (including means and standard deviations) were performed using Debra 5.2a software and Microsoft excel (some rounding up may have occurred in the tables as an inherent feature of the software).

10. Preparation of samples for quantification of radioactivity

The following methods were used:

i) Urine, cage wash and plasma: Aliquots of liquid samples were counted in Ultima Gold liquid scintillation cocktail (10mL) after stabilizing to ambient temperature and reduced light intensity.

ii) Blood: Weighed aliquots of cardiac blood (up to 0.2g) were combusted directly onto combusto pads contained in combusto cones.

iii) Liver, kidneys, brain, and skeletal muscle: Homogenized using an Ultra-Turrax fitted with an 8N shaft (aided by the addition of small amounts of HPLC grade water) where necessary). Portions of the homogenates were weighed into combustor-cones and combusted in a sample oxidizer following the addition of cellulose powder. The generated CO2 was trapped and mixed with a scintillation cocktail prior to radio assay.

iv) Skin, fur and carcass: Solubised in in a water bath containing alcoholic 2M potassium hydroxide) for 24 hours at 50˚C and added to scintillation cocktail prior to radio assay.

vi) All other tissues: Scissor minced portions or whole tissues were combusted directly onto combusto pads contained in combusto cones.

vii) Faeces: The collected faeces was homogenized with 50% of water (w/v). Aliquots of approximately 0.2g were combusted in a sample oxidizer

II. Results and Discussion

A. Recovery

The mean recovery of radioactivity from measurements in urine, faeces and tissues, in addition to carcass and GIT at sacrifice was approximately 95.09% and 98.20% the administered radioactivity in males and females respectively for the low dose group and 94.60% and 97.64% in males and females respectively for the high dose group. A summary of the radioactivity in percent of the administered dose found in excreta and in organs and tissues at sacrifice is presented in table 5.1.1- below.

Table 5.1.1-10: Recovery of radioactivity in excreta and body of rats following oral dosing of [phenyl-U-^{14}C]-fluopicolide, data expressed as % of dose administered.

Dose, route	**10 mg/kg bw**		**10 mg/kg bw**		**100 mg/kg bw**		**100 mg/kg bw**	
Experiment	**single low dose**		**single low dose**		**single high dose**		**single high dose**	
Duration, sex	**168 h, 4 males**		**168 h, 4 females**		**168 h, 4 males**		**168 h, 4 females**	
	[% dose administered]		**[% dose administered]**		**[% dose administered]**		**[% dose administered]**	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Urine	10.30	3.73	13.09	3.34	5.39	2.35	6.58	1.00
Faeces	82.50	3.83	82.09	2.68	87.46	6.32	88.28	8.06
Cage wash	0.96	0.46	2.03	0.78	1.02	0.19	1.76	1.40
Tissue/organ	1.25	0.07	0.99	0.07	0.75	0.18	1.03	0.20
Balance	**95.09**	**0.53**	**98.20**	**4.52**	**94.60**	**7.53**	**97.64**	**8.84**

B. Absorption

Based on radioactivity eliminated via the urine, the oral absorption is estimated to be 11.55% and 14.08% in males and females respectively for the low dose group and 6.14% and 7.61% in males and females respectively for the high dose groups. However, it is not possible to accurately calculate an exact value for this study. Owing to the high levels of faecal elimination it is likely that a high proportion of the dose was absorbed and eliminated *via* the bile, therefore, the actual oral absorption value is potentially much higher.

C. Distribution

The amount of radioactivity remaining in the total body at sacrifice, 168 hours following dosing was low (approximately 1%) and relatively similar between sexes and doses. The amount of radioactivity in the tissues at sacrifice accounted for 1.25±0.07% of the administered dose in males and 0.99±0.07% in females for the single low dose group. The corresponding values for the high dose group were 0.75±0.18% and 1.03±0.20% of the administered dose in males and females respectively.

In both sexes in the low and high dose groups, the highest concentrations of radioactivity were found in the liver and kidneys, and also in the skin and fur of females only of the high dose group. Thyroids in the low and high dose groups and bone and marrow in the high dose group were found to have levels of radioactivity below the limit of quantification. There were no marked differences between males and females in any organs in the low dose group; however, in the high dose group females displayed a generally higher concentration of radioactivity than males in each individual organ (with the exception of the intestine and contents). Table 5.1.1- shows the radioactivity in the various organs and tissues at sacrifice expressed as µg equivalents of [phenyl-U-14C]-fluopicolide.

Table 5.1.1- 11: Radioactive residues in organs and tissues at sacrifice expressed as µg equivalents of [phenyl-U-^{14}C]-fluopicolide

Dose, route	**10 mg/kg bw**		**10 mg/kg bw**		**100 mg/kg bw**		**100 mg/kg bw**	
Experiment	**single low dose**		**single low dose**		**single high dose**		**single high dose**	
Duration, sex	**168 h, 4 males**		**168 h, 4 females**		**168 h, 4 males**		**168 h, 4 females**	
	[µg equivalents]		**[µg equivalents]**		**[µg equivalents]**		**[µg equivalents]**	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Cardiac blood	0.120	0.010	0.183	0.020	0.389	0.151	0.596	0.086
Intestine[1]	0.075	0.037	0.036	0.008	0.184	0.172	0.139	0.040
Harder's gland	0.151	0.020	0.117	0.017	0.490	0.055	0.687	0.044
Carcass	0.095	0.004	0.085	0.005	0.507	0.185	0.549	0.050
Skin and fur	0.095	0.027	0.080	0.006	0.512	0.046	1.555	0.681
Cardiac plasma	0.019	0.006	0.015	0.005	0.024	0.048	0.059	0.040
Eyes	0.017	0.002	0.014	0.003	0.017	0.034	0.080	0.010
Brain	0.027	0.003	0.023	0.002	0.088	0.020	0.093	0.064
Fat	0.029	0.006	0.050	0.005	0.132	0.155	0.199	0.051
Heart	0.149	0.012	0.129	0.005	0.403	0.039	0.704	0.093
Lungs	0.056	0.004	0.075	0.009	0.207	0.095	0.298	0.065
Spleen	0.043	0.007	0.066	0.010	0.069	0.138	0.185	0.130
Liver	0.429	0.039	0.305	0.038	1.150	0.060	1.753	0.119
Kidneys	0.314	0.036	0.253	0.056	0.876	0.059	1.375	0.167
Thyroids	n.d	n.a	n.d	n.a	n.d	n.a	n.d	n.d
Ovaries	n.a	n.a	0.044	0.007	n.a	n.a	0.217	0.019
Testes	0.025	0.003	n.a	n.a	0.058	0.040	n.a	n.a
Pancreas	0.078	0.008	0.071	0.020	0.180	0.124	0.420	0.131
Adrenal	0.140	0.023	0.156	0.041	0.465	0.091	0.711	0.080
Uterus	n.a	n.a	0.016	0.001	n.a	n.a	0.073	0.085
Muscle	0.092	0.005	0.076	0.008	0.192	0.019	0.383	0.070
Stomach[1]	0.016	0.010	0.031	0.010	0.067	0.046	0.200	0.040
Bone & marrow	0.021	0.004	0.019	0.001	n.d	n.a	n.d	n.a

[1]Including contents, n.d = no detected radioactivity (twice background levels), n.a =not applicable

The potential for [phenyl-U-14C]-fluopicolide to accumulate in the tissues was measured by tissue/plasma and blood/tissue ratios. The highest blood plasma ratios in the low dose group were in the liver and kidneys, reaching 24.55 and 18.09 respectively for the males and 22.37 and 18.58 for the females. For several other tissues in the low dose group tissue/plasma ratios ranged from 2-8 in males (adrenals, heart and harder's gland)and from 3 to 13 (cardiac blood) in females, suggesting that some slight accumulation may have occurred in these tissues. In the high dose group only one male and three females had detectable values in the plasma; in the females the highest ratios were found in the liver, kidneys and skin & fur.

For both dose groups the high blood/plasma indicated that the test substance was present in greater quantities in the blood cells than in the plasma indicating that the tissue/blood ratio more accurately reflects the equilibrium between blood and tissue compartments. By using these measurements, in the low dose group only the liver (3.62), kidneys (2.64), harder's gland (1.28), heart (1.26) and adrenals (1.17) gave a ratio above one in males and liver (1.68) and kidneys (1.38) in females. The same tissues gave a ratio above one in males and females of the high dose group, with liver showing the highest ratio in males (3.21) and females (2.97). These results would therefore suggest that no accumulation has occurred in tissues.

D. Excretion

The excretion was fast and essentially complete 24-48 hours post administration. In males and females of both dose groups, the majority of the elimination of [phenyl-U-14C]-fluopicolide was via the faeces, with a corresponding lower amount being excreted via the urine (see table 5.1.1 below). The mean level of faecal elimination in the low dose group was 82.58% and 82.09% of the administered dose in males and females respectively and 87.46% and 88.28% in males and females of the high dose groups. The corresponding levels of mean radioactivity recovered in the urine for males and females respectively were 10.30% and 13.09% in the low dose group and 5.39% and 6.58% in the high dose group. A slight difference was therefore apparent in the excretion profiles of the low and high dose groups. Only a small amount of radioactivity was recovered in the body of the rats at sacrifice, suggesting that excretion was essentially complete at sacrifice.

Table 5.1.1- 12: Excretion of radioactivity at time intervals, expressed as % dose administered

Dose, route	10 mg/kg bw		10 mg/kg bw		100 mg/kg bw		100 mg/kg bw	
Experiment	single low dose		single low dose		single high dose		single high dose	
Duration, sex	168 h, 4 males		168 h, 4 females		168 h, 4 males		168 h, 4 females	
Time period (h)	[µg equivalents]		[µg equivalents]		[µg equivalents]		[µg equivalents]	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Radioactivity in faeces (% of dose administered, cumulative)								
0-24	53.91	24.64	72.54	7.78	79.79	6.25	78.59	8.48
0-48	76.76	7.06	81.31	2.58	85.79	6.07	87.29	7.94
0-72	80.88	4.58	81.82	2.69	86.82	6.20	87.99	7.89
0-96	82.02	4.02	81.95	2.67	87.22	6.27	88.20	8.07
0-120	82.36	3.88	82.02	2.68	87.35	6.30	88.24	8.07
0-144	82.50	3.85	82.06	2.68	87.43	6.32	88.26	8.06
0-168	82.58	3.83	82.09	2.68	87.46	6.32	88.28	8.06
Radioactivity in urine (% of dose administered, cumulative)								
0-6	0.83	0.53	2.10	0.37	0.55	0.21	0.54	0.16
0-24	4.85	1.32	9.35	1.06	2.94	1.21	3.62	1.09
0-48	8.36	2.91	12.29	3.02	4.42	1.73	5.73	0.96
0-72	9.45	3.41	12.72	3.26	4.95	2.02	6.24	0.96
0-76	9.89	3.59	12.88	3.30	5.19	2.22	6.39	0.99
0-120	10.09	3.67	12.97	3.32	5.29	2.30	6.48	0.99
0-144	10.21	3.71	13.04	3.33	5.35	2.34	6.54	1.00
0-168	10.30	3.73	13.09	3.34	5.39	2.35	6.58	1.00

III. Conclusions

The behavior of [phenyl-U-14C]-fluopicolide in male and female rats following a single oral low or high dose, can be characterized by the following observations:

- Oral absorption, based on renal excretion, was estimated to be 11.55% and 14.08% in males and females of the low dose group and 6.14% and 7.61% in males and females of the high dose group; however, the high rate of faecal elimination suggests that the majority of the dose was absorbed and excreted via the bile.
- Excretion was fast and almost complete at 48-hours post dose. The main route of excretion was via the faeces (82.58% and 82.09% in males and females of the low dose group and 87.46% and 88.28% in males and females of the high dose group). Only approximately 1% of the administered dose remained in the body at sacrifice, reflecting the high and fast rate of excretion.
- The majority of radioactivity in the tissues at sacrifice was found in the liver and kidneys of both sexes in the low and high dose groups, and additionally in the skin and fur of females in the high dose group.
- Analysis of the blood/tissue ratios showed that accumulation in the tissues was unlikely.

Assessment and conclusion by applicant:
An acceptable study yielding valid conclusions

Data Point:	KCA 5.1.1/03
Report Author:	[redacted]
Report Year:	2001
Report Title:	[Pyridyl-2,6 -14C]-AE C638206 - Single oral low dose rat A.D.E. study
Report No:	C012385
Document No:	M-202609-02-1
Guideline(s) followed in study:	EU (=EEC): 87/302/EEC Part B; JMAF: 59 NohSan No.4200; USEPA (=EPA): OPPTS 870.7485
Deviations from current test guideline:	not specified
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

The absorption, distribution, and excretion of fluopicolide were investigated with radiolabelled fluopicolide ([2,6-pyridyl-14C]-fluopicolide).

A single oral gavage dose of [2,6-pyridyl-14C]-fluopicolide was administered to male and female Sprague Dawley rats at a nominal dose of 10 mg/kg bw (Single Oral Low Dose). Urine and faeces were collected at regular intervals from dosing until sacrifice at 168-hours and the radioactivity in the samples determined by liquid scintillation counting (LSC). At sacrifice, the organs, tissues, blood and carcass were collected, and the radioactivity measured by LSC.

Recovery was high, with 93.87% (± 6.78%) in males and 95.88% (±1.33%) in females of the administered dose being recovered in the excreta along with remaining tissues and carcass at sacrifice.

Excretion was fast and almost complete at 48-hours post-dose. The main route of elimination was the faeces; the mean level of faecal elimination was 72.37% and 68.78% of the administered dose for males and females respectively. The corresponding mean recoveries of radioactivity in the urine for male and female rats respectively were 18.81% and 21.37%.

The amount of radioactivity remaining in the total body at sacrifice (168 hours following dosing) was low (less than 1% of the administered dose) and relatively similar between sexes. The greatest amounts of radioactivity were found in the liver, kidneys and blood of males and females. There was a slight sex difference observed with regard to the amount of radioactivity found in the spleen and intestines. Radioactivity in the thyroids of both sexes was below the limit of detection (twice background level).

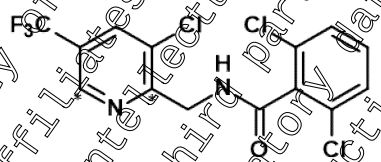
The highest blood plasma ratios were in the liver, reaching 6.61 and 8.42 in males and females respectively, and ranging from 1.6 to 6.9 in several other tissues, suggesting that some slight accumulation may have occurred in these tissues. However, owing the high blood/plasma ratio the tissue/blood ratio is considered to reflect the equilibrium between blood and tissue compartments more accurately. By using these measurements, no female tissue presented a ratio above 1, whilst in males only the liver and intestine & contents were slightly above 1 (1.06 and 1.25 respectively). These results would therefore suggest that no accumulation has occurred in tissues.

Based on renal excretion (plus radioactivity remaining in the tissues at sacrifice), the oral absorption of [2,6-pyridyl-14C]-fluopicolide is estimated to be 21.5% and 27.1% in males and females, respectively. However, owing to the large amount of faecal excretion observed, it is likely that a large proportion of the dose was absorbed and excreted *via* the bile, thus making the actual oral absorption value much higher.

I. Materials and methods

A. Materials

1. Test Material:

IUPAC Name	2,6-dichloro-N-[[3-chloro-5-(trifluoromethyl)-2-pyridinyl]methyl]-benzamide
CAS Name	benzamide, 2,6-dichloro-N-[[3-chloro-5-(trifluoromethyl)-2-pyridinyl]methyl]
Code name	AE C638206
Common name	Fluopicolide
Empirical formula	$C_{14}H_8Cl_3F_3N_2O$
CAS Number	239110-15-7
Molar mass	383.59 g/mol
Chemical structure	 * *denotes position of label*
Radiolabelled test material	[2,6-pyridyl-^{14}C]-fluopicolide*
Lot number	903-AE-3
Radiochemical purity	> 99% (by TLC & HPLC)
Specific radioactivity	62 mCi/mmole or 2.298 GBq/mmole
Specific radioactivity of dose suspensions	Checked by HPLC and radio assay
Stability of test compound	Stable at -20˚C

*referred to as [Pyridyl-2,6-14C]-Acyl picolide in the report

2. Vehicle: Aqueous methylcellulose (0.75% w/w)

3. Test animals:

Species: Rat
Strain: Sprague Dawley, CD
Age: Not stated
Weight at dosing: 168-187 g
Source: [redacted]
Acclimation period: Not stated
Identification: Ear tattoo
Diet: Certified rodent diet M20 (Pietrement, Provins, France)
Water: Provided *ad libitum* (filtered, softened water from municipal supply)
Housing: Housed in metal wire-mesh bottomed cages in metal trays containing absorbent paper during acclimatisation and in specialised metabolism cages thereafter

Environmental conditions:
Temperature: 22 ± 2˚C
Humidity: 55 ± 15%
Photoperiod: 12 hours
Air changes: Average 15 changes/hour

4. Preparation of dosing solutions

The doses were prepared by mixing weighed amounts of radiolabelled and non-radiolabelled material in acetonitrile to achieve a homogenous solution at the desired specific radioactivity. The solution was then gently evaporated under a stream of nitrogen, appropriate weights of aqueous methyl cellulose (0.75% w/w) added and the resulting solution ground to produce a suspension. The dose suspension was of sufficient quantity and concentration to provide a dose of 5 g per kg rat body weight. The dose suspensions were prepared on the day of dosing and assayed by HPLC for active ingredient content and for radioactivity content (by LSC) before, during and following the dosing procedure.

B. Study design and methods

1. In life dates: October 3, 2000 to November 10, 2000

2. Dose regimen and design of tests

Table 5.1.1- :Study design

Administered single dose of [2,6-pyridyl-^{14}C]-fluopicolide, route (experiment)	Number of rats and sex	Collection of samples during the test and at sacrifice	Duration
10 mg/kg bw, oral (single low dose)	4 males 4 females	Urine, faeces, cage wash, blood, plasma, organs, tissues, carcass	7 days

The rats each received a single dose of [2,6-pyridyl-U-^{14}C]-fluopicolide at a target dose of 10 mg/kg bw/d (low dose). Urine and faeces samples were collected at intervals until sacrifice at 168-hours, when the blood, organs, tissues and carcass were collected.

3. Dosing

Adequate volumes of the suspension of the test compound in aqueous methyl cellulose (0.75% w/w) were administered to fasted (18-hours) rats *via* a single oral gavage dose at a nominal low dose of 10 mg/kg bw. The doses for each individual animal were calculated based on the weights of the animal prior to dosing (food was replaced one-hour post dose and remained available *ad libitum* throughout the remainder of the study).

4. Collection of excreta

Following administration of the radiolabelled test substance, the animals were kept in specialized individual metabolism units (Jencon's metabowls MK III), which permitted the separate quantitative collection of urine and faeces, for a maximum of 168-hours following dosing. Both urine and faeces were collected frozen over solid CO_2 in weighed polystyrene boxes; carded cotton protected the samples from light.

Urine was collected at 0-6 and 6-24 hours and subsequently at 24-hour intervals following dosing, whilst faeces was collected at 24-hour intervals following dosing. Cages were washed with distilled water for each 24-hour period post-dose and then with acetonitrile at the end of the in-life phase; cage washes were retained for analysis. The radioactivity content was determined by HPLC.

5. Blood and plasma

Cardiac blood samples were taken from each animal at sacrifice, and plasma prepared by centrifugation (approximately 2000 x g for 10 minutes).

6. Sacrifice

All animals were killed by exsanguination under Imalgene 500 anesthesia, at 7 days following dose administration.

7. Tissues and organs collected at sacrifice

The following organs and tissues were collected at sacrifice: Liver, kidney, heart, lungs, brain, spleen, pancreas, fat (abdominal), muscle, eyes, ovaries, testes, intestinal tract plus contents, stomach plus contents, bone and marrow, adrenals, uterus, thyroid, harderian glands, skin and fur. The residual carcass was retained for analysis. Samples were processed immediately if possible or were frozen until required.

8. Measurement of radioactivity

Radioactivity was measured by liquid scintillation counting with automatic external standard quench correction. The limit of detection was taken to be twice the background values for blank samples in appropriate scintillation cocktails.

9. Data processing

The calculations of % dose recovery and compound concentrations (including means and standard deviations) were performed using Debra 5.2a software and Microsoft excel (some rounding up may have occurred in the tables as an inherent feature of the software).

10. Preparation of samples for quantification of radioactivity

The following methods were used:

i) Urine, cage wash and plasma: Aliquots of liquid samples were counted in Ultima Gold liquid scintillation cocktail (10mL) after stabilizing to ambient temperature and reduced light intensity.

ii) Blood: Weighed aliquots of cardiac blood (up to 0.2g) were combusted directly onto combusto pads contained in combusto cones.

iii) Skin, fur and carcass: Solubised in in a water bath containing alcoholic 2M potassium hydroxide) for 24 hours at 50 °C and added to scintillation cocktail prior to radioassay.

iv) Tissues: Scissor minced portions (fat, testes, bone, marrow, uterus) or whole tissues (ovaries, eyes, Harderian glands, thyroid and adrenals) were combusted directly onto combusto pads contained in combusto cones. Other tissues were homogenized using an Ultra-Turrax fitted with an 8N shaft (aided by the addition of small amounts of HPLC grade water) where necessary). Portions of the homogenates were weighed into combustor-cones and combusted in a sample oxidizer following the addition of cellulose powder. The generated CO2 was trapped and mixed with a scintillation cocktail prior to radio assay.

v) Faeces: The collected faeces was homogenized with approximately twice their volume of water. Aliquots of approximately 0.2g were combusted in a sample oxidizer following the addition of a small quantity of cellulose powder.

II. Results and Discussion

B. Recovery

The mean recovery of radioactivity from measurements in urine, faeces and tissues, in addition to carcass and GIT at sacrifice was approximately 93.87% (± 6.78%) and 95.88% (±1.33%) of the administered radioactivity in males and females, respectively. A summary of the radioactivity in percent of the administered dose found in excreta and in organs and tissues at sacrifice is presented in table 5.1.1- 13 below.

Table 5.1.1- 13: Recovery of radioactivity in excreta and body of rats following oral dosing of [2,6-pyridyl-14C]-fluopicolide, data expressed as % of dose administered.

Dose, route	**10 mg/kg bw**		**10 mg/kg bw**	
Experiment	**single low dose**		**single low dose**	
Duration, sex	**168 h, 4 males**		**168 h, 4 females**	
	[% dose administered]		**[% dose administered]**	
	Mean	SD	Mean	SD
Urine	18.81	1.48	21.37	4.64
Faeces	72.37	5.12	68.78	4.70
Cage wash	2.04	1.12	5.27	1.24
Tissue/organ	0.66	0.37	0.46	0.03
Balance	**93.87**	**6.78**	**95.88**	**1.33**

B. Absorption

Based on radioactivity eliminated via the urine (and that remaining in the tissues), the oral absorption of [2,6-pyridyl-14C]-fluopicolide is estimated to be 22.5% and 27.1% in males and females respectively; however, it is not possible to accurately calculate an exact value for this study. Owing to the high levels of faecal elimination it is likely that a high proportion of the dose was absorbed and eliminated *via* the bile, therefore, the actual oral absorption value is potentially much higher.

C. Distribution

The amount of radioactivity remaining in the total body at sacrifice, 168 hours following dosing was low (less than 1%) and relatively similar between the sexes. The amount of radioactivity in the tissues at sacrifice accounted for 0.66±0.37% of the administered dose in males and 0.46±0.03% in females.

In both sexes, the highest concentrations of radioactivity (µg equivalents of [2,6-pyridyl-14C]-fluopicolide) were found in the liver, kidneys and blood. There was a slight difference between males and females in the spleen and intestines (with contents); the radioactivity found in the female spleen was 0.107 µg/g (compared with 0.048 µg/g in males), whilst the concentration of radioactivity in the male intestine & contents was 0.230 µg/g compared with 0.027 µg/g in females. The amount of radioactivity in the thyroids of both sexes was below the limit of quantification. Table 5.1.1- shows the radioactivity in the various organs and tissues at sacrifice expressed as µg equivalents of [2,6-pyridyl-14C]-fluopicolide.

Table 5.1.1- 14: Radioactive residues in organs and tissues at sacrifice expressed as µg equivalents of [2,6-pyridyl-14C]-fluopicolide

Dose, route	**10 mg/kg bw**		**10 mg/kg bw**	
Experiment	**single low dose**		**single low dose**	
Duration, sex	**168 h, 4 males**		**168 h, 4 females**	
	[µg equivalents]		**[µg equivalents]**	
	Mean	SD	Mean	SD
Cardiac blood	6.194	2.745	18.667	13.449
Intestine[1]	5.750	1.500	7.625	1.109
Harder's gland	0.625	0.285	1.417	0.788
Carcass	0.778	0.272	1.375	0.759
Skin and fur	1.708	0.966	5.042	2.270
Cardiac plasma	1.000	0.000	1.000	0.000
Eyes	0.028	0.056	0.125	0.160
Brain	0.028	0.056	0.625	0.438
Fat	0.542	0.417	1.667	1.054
Heart	1.819	1.103	3.125	2.140
Lungs	2.014	1.366	5.250	3.248
Spleen	1.819	0.933	6.417	4.193
Liver	6.611	3.636	8.417	5.940
Kidneys	3.806	1.456	6.917	4.764
Thyroids	n.d	n.a	n.d	n.a
Ovaries	n.a	n.a	1.833	1.374
Testes	0.639	0.918	n.a	n.a
Pancreas	0.417	0.419	1.5836	1.167
Adrenal	1.792	0.854	3.667	2.261
Uterus	n.a	n.a	0.750	0.319
Muscle	0.514	0.344	0.625	0.438
Stomach[1]	0.236	0.224	0.875	0.832
Bone & marrow	1.347	1.778	0.667	0.385

[1]including contents, n.d = not detected (below twice background), n.a = not applicable

The potential for [phenyl-U-14C]-fluopicolide to accumulate in the tissues was measured by tissue/plasma and blood tissue ratios. The highest blood/plasma ratios were in the liver, reaching 6.61 and 8.42 in males and females respectively. For several other tissues (intestine & contents, kidney heart, lungs, spleen and skin & fur in both sexes plus the adrenals in females) tissue/plasma ratios ranged from 1.6 to 6.9, suggesting that some slight accumulation may have occurred in these tissues. However, these tissues are highly irrigated, and the radioactivity could be owing to the presence of remnant blood in these tissues. Furthermore, the high blood/plasma indicated that the test substance was present in greater quantities in the blood cells than in the plasma and so the tissue/blood ratio would more accurately reflect the equilibrium between blood and tissue compartments. By using these measurements, no female tissue presented a ratio above 1, whilst in males only the liver and intestine & contents were slightly above 1 (1.06 and 1.25 respectively). These results would therefore suggest that no accumulation has occurred in tissues.

D. Excretion

The excretion was fast and almost complete by 48-hours post administration. In males and females, the majority of the elimination of [2,6-pyridyl-14C]-fluopicolide was via the faeces, with a corresponding lower amount being excreted via the urine (see table 5.1.1 below). The mean level of faecal elimination over the seven-day study period was 72.37% and 68.78% of the administered dose in males and females respectively. The corresponding levels of mean radioactivity recovered in the urine for males and females respectively were 18.81% and 21.37% of the administered dose. Therefore, there was no apparent difference in the elimination profile between sexes. Only a small amount of radioactivity was recovered in the body of the rats at sacrifice 9less than 1% of the administered dose), suggesting that excretion was essentially complete at sacrifice.

Table 5.1.1- 15: Excretion of radioactivity at time intervals following a single oral low dose of [2,6-pyridyl-14C]-fluopicolide, expressed as % dose administered

Dose, route	**10 mg/kg bw**		**10 mg/kg bw**	
Experiment	**single low dose**		**single low dose**	
Duration, sex	**168 h, 4 males**		**168 h, 4 females**	
Time period (h)	**[µg equivalents]**		**[µg equivalents]**	
	Mean	SD	Mean	SD
Radioactivity in faeces (% of dose administered, cumulative)				
0-24	50.15	6.36	55.98	6.78
0-48	62.72	6.32	66.64	4.50
0-72	67.12	5.79	68.10	4.67
0-96	69.42	4.67	68.49	4.71
0-120	71.65	5.42	68.67	4.70
0-144	72.10	5.24	68.73	4.70
0-168	72.37	5.12	67.78	4.70
Radioactivity in urine (% of dose administered, cumulative)				
0-6	5.76	1.65	3.32	1.27
0-24	12.88	3.10	13.31	4.03
0-48	16.22	1.62	19.51	4.57
0-72	17.43	1.55	20.74	4.56
0-76	18.15	1.46	21.13	4.64
0-120	18.51	1.44	21.28	4.63
0-144	18.70	1.46	21.33	4.64
0-168	18.81	1.48	21.37	4.64

III. Conclusions

The behaviour of [2,6-pyridyl-14C]-fluopicolide in male and female rats following a single oral low dose, can be characterized by the following observations:

- Oral absorption, based on renal excretion (plus radioactivity in tissues at sacrifice), was estimated to be 21.5% and 27.1% in males and females respectively; however, the high rate of faecal elimination suggests that the majority of the dose was absorbed and excreted via the bile.
- Excretion was fast and almost complete at 48-hours post dose. The main route of excretion was via the faeces (72.37% and 68.78% in males and females respectively). Only approximately 1% of the administered dose remained in the body at sacrifice, reflecting the high and fast rate of excretion.
- The majority of radioactivity in the tissues in males and females at sacrifice was found in the liver, kidneys and blood. There was a slight sex difference in the radioactivity found in the spleen and intestine & contents.
- Analysis of the blood/tissue ratios showed that accumulation in the tissues was unlikely.

Assessment and conclusion by applicant:

An acceptable study yielding valid results.

Data Point:	KCA 5.1.1/04
Report Author:	[redacted]
Report Year:	2002
Report Title:	Rat bile excretion study (phenyl-U-14C)-AE C638206
Report No:	C021984
Document No:	M-212243-01-1
Guideline(s) followed in study:	EU (=EEC): 87/302/EEC Part B; JMAF: 59 NohSan No 4200; USEPA (=EPA): OPPTS 870.7485
Deviations from current test guideline:	not specified
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

The absorption, distribution, and excretion of fluopicolide were investigated in bile cannulated rats with radiolabelled fluopicolide ([Phenyl U-14C]-fluopicolide).

A single oral gavage dose of [Phenyl U-14C]-fluopicolide was administered to bile cannulated male and female Sprague Dawley rats at a nominal dose of 10 mg/kg bw (Single Oral Low Dose) or 100 mg/kg bw (Single Oral High Dose). Urine, bile and faeces were collected at regular intervals from dosing until sacrifice at 48-hours and the radioactivity in the samples determined by liquid scintillation counting (LSC). At sacrifice, the intestine & contents, stomach & contents, blood and carcass were collected, and the radioactivity measured by LSC.

Recovery was high, with 98.85 ± 3.45% and 102.26±2.33% of the administered dose being recovered in low dose males and females and 93.54±1.57% and 97.04±4.38% being recovered in high dose males and females respectively.

Excretion was fast and essentially complete at 48-hours post-dose. The majority of radioactivity in the low dose group was excreted via the bile (followed by faeces and then urine), whilst in the high dose group the main route of elimination was via the faeces (followed by bile and then urine). The amount of radioactivity recovered in the bile, faeces and urine from the low dose group was 70.02% and 73.88%, 21.48% and 19.28% and 4.55% and 6.715 of the administered dose for males and females respectively. The corresponding mean recoveries of radioactivity in the bile, faeces and urine for high dose male and female rats respectively were 31.34% and 31.93%, 59.37% and 55.72% and 1.42% and 5.82%.

A comparison of the rates of elimination in the urine, cage wash, bile and faeces in this biliary excretion study and the previous ADE study showed that a higher amount of radioactivity was excreted via the urine in non-cannulated rats compared with cannulated rats, and that the amount of recovery overall was higher in the bile cannulated rats. This suggest that a proportion of the dose excreted via the bile is reabsorbed and excreted via the urine and/or faeces (enterohepatic circulation).

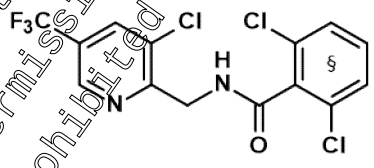
The amount of radioactivity remaining in the total body at sacrifice (168 hours following dosing) was low (approximately 2%) and relatively similar between sexes and doses. The greatest amounts of radioactivity were found in the residual carcass and the intestinal contents.

The estimated oral absorption, based on recoveries in urine (plus cage wash), bile and tissues (with the exclusion of the intestine and stomach contents) was 80.06 % of the administered dose in the low dose group (77.24% in males and 82.88% in females) and 37.30% of the administered dose in the high dose group (33.77% in males and 40.83% in females).

I. Materials and methods

A. Materials

1. Test Material:

IUPAC Name	2,6-dichloro-N-[[3-chloro-5-(trifluoromethyl)-2-pyridinyl]methyl]-benzamide
CAS Name	benzamide, 2,6-dichloro-N-[[3-chloro-5-(trifluoromethyl)-2-pyridinyl]methyl]
Code name	AE C638206
Common name	Fluopicolide
Empirical formula	$C_{14}H_8Cl_3F_3N_2O$
CAS Number	239110-15-7
Molar mass	383.59 g/mol
Chemical structure	 □ *denotes position of label*
Radiolabelled test material	[phenyl-U-^{14}C]-fluopicolide*
Lot number	CFQ12747
Radiochemical purity	99.1% (by HPLC)
Specific radioactivity	58.05 mCi/mmole or 2148 MBq/mmole
Specific radioactivity of dose suspensions	Checked by HPLC and radio assay
Stability of test compound	Stable at -20˚C

*referred to as [phenyl-U-^{14}C]-AE C638206 in the report

2. Vehicle: Aqueous methyl cellulose (0.75% w/w)

3. Test animals:

Species:	Rat
Strain:	Sprague Dawley
Age:	Not stated
Weight at dosing:	230.95 - 338.60g
Source:	[redacted]
Acclimation period:	Not stated
Identification:	Ear tattoo
Diet:	Certified rodent diet A04C (UAR, Villemoisson, France)
Water:	Provided *ad libitum* (filtered, softened water from municipal supply)
Housing:	Housed in metal wire-mesh bottomed cages in metal trays containing absorbent paper during acclimatisation and in specialised metabolism cages thereafter
Environmental conditions	
Temperature:	22 ± 2˚C
Humidity:	55 ± 15%
Photoperiod:	12 hours
Air changes	Average 15 changes/hour

4. Preparation of dosing solutions

The doses were prepared by mixing weighed amounts of radiolabelled and non-radiolabelled material in acetonitrile to achieve a homogenous solution at the desired specific radioactivity. The solution was then gently evaporated under a stream of nitrogen, appropriate weights of aqueous methyl cellulose (0.75% w/w) added and the resulting solution ground to produce a suspension. The dose suspension was of sufficient quantity and concentration to provide a dose of 1g per 200 g rat body weight. The dose suspensions for the low and high dose groups were prepared on the day of dosing and assayed by HPLC for active ingredient content and for radioactivity content (by LSC) before, during and following the dosing procedure.

B. Study design and methods

1. In life dates: October 25, 2001 to February 12, 2002

2. Dose regimen and design of tests

Table 5.1.1- 16: Study design

Administered single dose of [phenyl-U-^{14}C]-fluopicolide , route (experiment)	Number of rats and sex	Collection of samples during the test and at sacrifice	Duration
10 mg/kg bw, oral (single low dose)	4 males 4 females	Urine, faeces, cage wash, bile, blood, plasma, organs & tissues*, carcass	48-hours
100 mg/kg bw, oral (single low dose)	4 males 4 females	Urine, faeces, cage wash, bile, blood, plasma, organs & tissues*, carcass	48-hours
* Comprises intestine & contents and stomach & contents			

The rats each received a single dose of [phenyl-U-14C]-fluopicolide at a target dose of either 10 mg/kg bw/d (low dose) or 100 mg/kg bw/d (high dose). Urine, faeces and bile samples were collected at intervals until sacrifice at 48-hours, when the blood, GI tract and carcass were collected.

3. Bile cannulation surgery

Surgery was performed 24-hours prior to dosing. Male and female rats were anaesthetized with Imalgène-500 and the bile ducts cannulated through a mid-line incision. The cannulae were exteriorized dorsally and the body wall and sutured with surgical thread. Following surgery, the cannulae of each animal was led through the roof of the individual glass metabolism cage, permitting the collection of the bile into a weighed container. Animals showing a normal flow of bile were selected for the study.

4. Dosing

Adequate volumes of the suspension of the test compound in aqueous methyl cellulose (0.75% w/w) were administered to fasted (16-hours), cannulated rats *via* a single oral gavage dose.

5. Collection of excreta

Following administration of the radiolabelled test substance, animals of the high and low dose groups were housed in glass metabolism cages (Jencon's metabowls MK III), which permitted the separate quantitative collection of urine and faeces, for a maximum of 48-hours following dosing. Bile was individually collected into an appropriate weighed container, via each animals cannulae which was led through the roof of the metabolism cage to avoid disturbing the animal during collection

Urine and bile were collected at 0-6-, 6-24- and 24-48-hours following dosing, whilst faeces was collected at 24-hour intervals following dosing. Cages were washed with distilled water for each 24-hour period post-dose and then with acetonitrile at the end of the in-life phase; cage washes were retained for analysis. Samples were processed immediately if possible or were frozen until required.

6. Blood and plasma

Cardiac blood samples were taken from each animal at sacrifice, and plasma prepared by centrifugation (approximately 1000 x g for 10 minutes).

7. Sacrifice

All animals were killed by exsanguination under Imogene 500 anaesthesia, at 48-hours following dose administration.

8. Tissues and organs collected at sacrifice

The intestinal tract, intestinal tract contents, stomach and stomach contents were removed from each animal following sacrifice. The residual carcass was retained for analysis. Samples were processed immediately if possible or were frozen until required.

9. Measurement of radioactivity

Radioactivity was measured by liquid scintillation counting with automatic external standard quench correction. The limit of detection was taken to be twice the background values for blank samples in appropriate scintillation cocktails.

10. Data processing

The calculations of % dose recovery and compound concentrations (including means and standard deviations) were performed using Debra 5.2 software and Microsoft excel (some rounding up differences may have occurred in the tables as an inherent feature of the software).

11. Preparation of samples for quantification of radioactivity

The following methods were used:

i) Urine, cage wash, bile and plasma: Aliquots of liquid samples were counted in Ultima Gold liquid scintillation cocktail (10mL) after stabilizing to ambient temperature and reduced light intensity.

ii) Blood: Weighed aliquots of cardiac blood (up to 0.2g) were combusted directly onto combusto pads contained in combusto cones.

iii) GI tract: Homogenized using an Ultra-Turrax fitted with an 8N shaft (aided by the addition of small amounts of deionised water where necessary). Portions of the homogenates were weighed into combustor-cones and combusted in a sample oxidizer following the addition of cellulose powder. The generated CO2 was trapped and mixed with a scintillation cocktail prior to radio assay.

iv) Faeces: The collected faeces was homogenized with a portion of water. Samples of various weights were combusted in a sample oxidizer following the addition of a small quantity of cellulose powder. The generated CO2 was trapped and mixed with a scintillation cocktail prior to radio assay.

II. Results and Discussion

A. Recovery

The mean recovery of radioactivity from measurements in urine, bile and faeces, in addition to carcass and GIT at sacrifice was approximately 98.85% and 102.26% the administered radioactivity in males and females respectively for the low dose group and 93.54% and 97.04% in males and females respectively for the high dose group. A summary of the radioactivity in percent of the administered dose found in excreta and in organs and tissues at sacrifice is presented in table 5.1.1- below.

Table 5.1.1- 17 : Recovery of radioactivity in excreta and body of rats following oral dosing of [phenyl-U-^{14}C]-fluopicolide, data expressed as % of dose administered.

Dose, route	**10 mg/kg bw**		**10 mg/kg bw**		**100 mg/kg bw**		**100 mg/kg bw**	
Experiment	**single low dose**		**single low dose**		**single high dose**		**single high dose**	
Duration, sex	**48 h, 4 males**		**48 h, 4 females**		**48 h, 4 males**		**48 h, 4 females**	
	[% dose administered]		**[% dose administered]**		**[% dose administered]**		**[% dose administered]**	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Urine	4.55	4.34	6.71	1.82	1.42	0.62	5.82	3.76
Faeces	21.48	7.47	19.28	7.03	59.34	3.09	55.72	6.13
Cage wash	70.02	6.79	73.88	8.03	31.34	1.21	31.93	10.24
Bile	0.77	0.79	0.91	0.60	0.18	0.08	2.00	3.34
Tissues	2.03	0.40	1.48	0.16	1.25	0.23	1.57	0.44
Balance	**98.85**	**3.45**	**102.26**	**2.33**	**93.54**	**1.57**	**97.04**	**4.38**

B. Absorption

The estimated oral absorption, based on recoveries in urine (plus cage wash), bile and tissues (with the exclusion of the intestine and stomach contents) was 80.06 % of the administered dose in the low dose group (77.24% in males and 82.88% in females) and 37.30% of the administered dose in the high dose group (33.77% in males and 40.83% in females).

C. Distribution

The amount of radioactivity remaining in the total body at sacrifice, 48 hours following dosing was relatively low (approximately 2%) and relatively similar between sexes and doses. The amount of radioactivity in the tissues at sacrifice accounted for 2.027±0.403% of the administered dose in males and 1.483±0.165% in females for the single low dose group. The corresponding values for the high dose group were 1.270±0.235% and 1.574±0.443% of the administered dose in males and females respectively.

In both sexes of the low and high dose groups, the highest levels of radioactivity were found in the residual carcass and intestinal contents, with 1.780% and 1.268% of the administered dose being found in the residual carcass of low dose males and females respectively, and 0.754% and 0.984% of high dose males and females. In low dose males and females respectively 0.129% and 0.100% were found in the intestinal contents, whilst 0.440% and 0.487% of the administered dose was found in the intestinal contents of high dose males and females. In all other tissues, the radioactivity recovered was generally less than 0.06%, suggesting that in both dose groups and sexes, [phenyl-U-14C]-fluopicolide was completely absorbed and/or eliminated at 48-hours following dosing. Table 5.1.1- shows the radioactivity in the various organs and tissues at sacrifice expressed as µg equivalents of [phenyl-U-14C]-fluopicolide.

Table 5.1.1- 18: Radioactive residues in organs and tissues at sacrifice expressed as % administered dose

Dose, route	**10 mg/kg bw**		**10 mg/kg bw**		**100 mg/kg bw**		**100 mg/kg bw**	
Experiment	**single low dose**		**single low dose**		**single high dose**		**single high dose**	
Duration, sex	**48 h, 4 males**		**48 h, 4 females**		**48 h, 4 males**		**48 h, 4 females**	
	% administered dose		**% administered dose**		**% administered dose**		**% administered dose**	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Cardiac blood	0.055	0.017	0.062	0.029	0.017	0.008	0.025	0.009
Intestine content	0.129	0.148	0.100	0.036	0.040	0.248	0.487	0.255
Residual carcass	1.780	0.287	1.268	0.138	0.754	0.124	0.984	0.286
Cardiac plasma	0.008	0.005	0.003	0.001	0.003	0.001	0.002	0.001
Intestine	0.042	0.006	0.040	0.012	0.048	0.006	0.061	0.032
Stomach	0.011	0.003	0.010	0.002	0.005	0.000	0.006	0.001
Stomach content	0.001	0.002	n.a	n.a	0.003	0.001	0.007	0.006
Total	**2.027**	**0.403**	**1.483**	**0.165**	**1.270**	**0.235**	**1.574**	**0.443**

D. Excretion

There was a difference in the elimination pathway between the high and low dose groups, although no significant sex differences were observed within the dose groups (see table 5.1.1 below).

In the low group dose, the majority of the administered dose was excreted via the bile, followed by the faeces, with the lowest amount being found in the urine. The amounts excreted were similar between sexes with a slightly higher amount of radioactivity being recovered in the urine of female rats compared with males. The mean amounts of radioactivity recovered in males and females respectively were 70.02% and 73.88% in bile, 21.48% and 19.28% in faeces and 4.55% and 6.71% of the administered dose in urine. The total elimination of [phenyl-U-14C]-fluopicolide (bile, urine, faeces and cage wash) at 48-hours post-dose, was therefore 96.82% in males and 100.78% in females.

In the high dose group, the majority of the administered dose was eliminated via the faeces, followed by bile and then urine. The mean amounts of radioactivity recovered in males and females respectively were 59.34% and 55.72% in faeces, 31.4% and 31.93% in bile and 1.42% and 5.82% of the administered dose in urine. The apparent sex difference in the radioactivity recovered in the urine was a result of one female rat (17.46% of the administered dose eliminated via the urine compared with 1-2% in other high dose female rats). The total elimination of [phenyl-U-14C]-fluopicolide (bile, urine, faeces and cage wash) at 48-hours post-dose, was 92.27% in males and 95.47% in females.

Table 5.1.1- 19: Excretion of radioactivity at time intervals, expressed as % dose administered

Dose, route	10 mg/kg bw		10 mg/kg bw		100 mg/kg bw		100 mg/kg bw	
Experiment	single low dose		single low dose		single high dose		single high dose	
Duration, sex	48 h, 4 males		48 h, 4 females		48 h, 4 males		48 h, 4 females	
Time period (h)	[µg equivalents]		[µg equivalents]		[µg equivalents]		[µg equivalents]	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Radioactivity in faeces (% of dose administered, cumulative)								
0-24	19.55	8.35	14.81	8.27	51.00	5.31	27.25	9.22
0-48	21.48	7.47	19.28	7.03	59.34	3.09	55.72	6.13
Radioactivity in urine (% of dose administered, cumulative)								
0-6	0.57	0.20	0.69	0.14	0.13	0.08	0.27	0.38
0-24	3.62	3.61	5.75	1.81	0.94	0.43	3.40	4.07
0-48	4.55	4.34	6.71	1.82	1.42	0.62	5.82	4.76
Radioactivity in bile (% of dose administered, cumulative)								
0-6	21.64	8.08	20.12	5.97	7.19	1.36	5.20	0.99
0-24	65.51	5.59	70.81	7.28	26.90	1.56	26.02	10.05
0-48	70.02	6.79	73.88	8.03	31.34	1.21	31.93	10.24

In table 5.1.1.20 below, the amount of the administered radioactivity recovered in the urine, faeces and bile is compared with the recoveries measured in the urine and faeces (48-hours post-dose) in the previous ADE study (M-204781-01-1).

Table 5.1.1- 20: Comparison of the radioactivity recovered in excreta at 48-hours post-dose in the ADE and biliary excretion studies (expressed as % dose administered)

Dose, route	10 mg/kg bw		10 mg/kg bw		100 mg/kg bw		100 mg/kg bw	
Experiment	ADME single low dose		BK single low dose		ADME single high dose		BK high dose	
	[% dose administered]		[% dose administered]		[% dose administered]		[% dose administered]	
	Male	Female	Male	Female	Male	Female	Male	Female*
Urine & cagewash	9.14	13.95	5.32	7.62	5.27	7.26	1.60	7.82 (2.27)
Faeces	76.76	81.31	21.48	19.28	85.79	87.29	59.34	55.72 (58.57)
Bile	-	-	70.02	73.88	-	-	31.34	31.93 (36.82)
Balance	85.90	95.26	96.82	100.78	91.06	94.55	92.28	95.47 (97.66)

* ( ) = mean recoveries when animal LM2F191 is excluded

The levels of radioactivity in urine were greater for both doses and sexes in the ADME study than in the biliary excretion study; furthermore, the total amount of radioactivity excreted at 48-hours post-dose was higher in the biliary excretion study than in the ADE study. This discrepancy between cannulated and non-cannulated rats is likely to be a result of enterohepatic circulation.

III. Conclusions

The behaviour of [phenyl-U-14C]-fluopicolide in male and female bile cannulated rats following a single oral low or high dose, can be characterized by the following observations:

- Oral absorption, based on recoveries in urine (plus cage wash), bile and tissues (with the exclusion of the intestine and stomach contents) was 80.06 % of the administered dose in the low dose group (77.24% in males and 82.88% in females) and 37.30% of the administered dose in the high dose group (33.77% in males and 40.83% in females).
- Excretion was fast and essentially complete at sacrifice (48-hours post dose). The main route of excretion in the low dose group was via the bile (70.02% in males and 73.88% in females), followed by the faeces (21.48% in males and 19.28% in females) and the urine (4.55% in males and 6.71% in females). In the high dose group, the main route of excretion was via the faeces (59.34% in males and 55.72% in females), followed by bile (31.4% in males and 31.93% in females) and urine (1.42% in males and 5.82% in females). Only approximately 2% of the administered dose remained in the body at sacrifice, reflecting the high and fast rate of excretion.
- A comparison of the amount of radioactivity recovered in excreta with the previous ADME study suggests that some enterohepatic circulation of [phenyl-U-14C]-fluopicolide is occurring.
- The majority of radioactivity in the tissues at sacrifice was found in the residual carcass and intestinal contents of both sexes in the low and high dose groups.

Assessment and conclusion by applicant:
An acceptable study yielding valid conclusions

Data Point:	KCA 5.1.1/05
Report Author:	[redacted]
Report Year:	2003
Report Title:	Single oral low dose rat bile excretion study Code: (Pyridyl-2,6-14C)-AE C638206
Report No:	C032181
Document No:	M-230976-01-1
Guideline(s) followed in study:	EU (=EEC): 87/302/EEC, B; MHW (Japan): 59 NohSan No. 4200, 1985; USEPA (=EPA): OPPTS 870.7485, 1998
Deviations from current test guideline:	not specified
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

The absorption, distribution, and excretion of fluopicolide were investigated with radiolabelled fluopicolide ([2,6-pyridyl-^{14}C]-fluopicolide) in bile cannulated rats.

A single oral gavage dose of [2,6-pyridyl-^{14}C]-fluopicolide was administered to bile cannulated male and female Sprague Dawley rats at a nominal dose of 10 mg/kg bw (Single Oral Low Dose). Urine, bile and faeces were collected at regular intervals from dosing until sacrifice at 48-hours and the radioactivity in the samples determined by liquid scintillation counting (LSC). At sacrifice, the intestine & contents, stomach & contents, blood and carcass were collected, and the radioactivity measured by LSC.

Recovery was high, with 100.59 ± 2.80% and 103.63±7.87% of the administered dose being recovered in males and females respectively.

Excretion was fast and essentially complete at 48-hours post-dose. The majority of radioactivity was excreted via the bile, followed by faeces and then urine. The amount of radioactivity recovered in the bile, faeces and urine following a single oral low dose of ([2,6-pyridyl-^{14}C]-fluopicolide was 51.69% and 51.74%, 40.27% and 39.16% and 5.83% and 10.42% of the administered dose for males and females respectively.

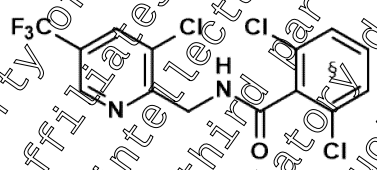
The amount of radioactivity remaining in the total body at sacrifice (168 hours following dosing) was low (approximately 2.21% in males at 0.8% in females) and relatively similar between sexes. The greatest amounts of radioactivity were found in the intestinal contents, with relatively low amounts (<0.01% to 0.58%). Therefore ([2,6-pyridyl-^{14}C]-fluopicolide was essentially completely absorbed and/or eliminated by 48-hours post-dose.

The estimated oral absorption, based on recoveries in urine (plus cage wash), bile and tissues (with the exclusion of the intestine and stomach contents) was 59±6.92% in males and 64.05±10.67% in females.

I. Materials and methods

A. Materials

1. Test Material:

IUPAC Name	2,6-dichloro-N-[[3-chloro-5-(trifluoromethyl)-2-pyridinyl]methyl]-benzamide
CAS Name	benzamide, 2,6-dichloro-N-[[3-chloro-5-(trifluoromethyl)-2-pyridinyl]methyl]
Code name	AE C638206
Common name	Fluopicolide
Empirical formula	$C_{14}H_8Cl_3F_3N_2O$
CAS Number	239110-15-7
Molar mass	383.59 g/mol
Chemical structure	 * denotes position of label
Radiolabelled test material	[2,6-pyridyl-^{14}C]-fluopicolide*
Lot number	903 AE-3
Radiochemical purity	99.1% (by HPLC)
Specific radioactivity	62 mCi/mmole or 2298 MBq/mmole
Specific radioactivity of dose suspensions	Checked by HPLC and radio assay
Stability of test compound	Stable at -20°C

*referred to as [Pyridyl-2,6-^{14}C]-AE C638206 in the report

2. Vehicle: Aqueous methyl cellulose (0.75% w/w)

3. Test animals:

Species: Rat
Strain: Sprague Dawley
Age: Not stated
Weight at dosing: 209.07 – 335.82g
Source: [redacted]
Acclimation period: Not stated
Identification: Ear tattoo
Diet: Certified rodent diet A04C (UAR, Villemoisson, France)
Water: Provided *ad libitum* (filtered, softened water from municipal supply)
Housing: Housed in metal wire-mesh bottomed cages in metal trays containing absorbent paper during acclimatisation and in specialised metabolism cages thereafter

Environmental conditions
Temperature: 22 ± 2°C
Humidity: 55 ± 15%
Photoperiod: 12 hours
Air changes: Average 15 changes/hour

4. Preparation of dosing solutions

The doses were prepared by mixing weighed amounts of radiolabelled and non-radiolabelled material in acetonitrile to achieve a homogenous solution at the desired specific radioactivity. The solution was then gently evaporated under a stream of nitrogen, appropriate weights of aqueous methyl cellulose (0.75% w/w) added and the resulting solution ground to produce a suspension. The dose suspension was of sufficient quantity and concentration to provide a dose of 1g per 200 g rat body weight. The dose suspensions for the low and high dose groups were prepared on the day of dosing and assayed by HPLC for active ingredient content and for radioactivity content (by LSC) before, during and following the dosing procedure.

B. Study design and methods

1. In life dates: May 23 2002 to July 1 2002

2. Dose regimen and design of tests

Table 5.1.1- 21:Study design

Administered single dose of [2,6-pyridyl-14C]-fluopicolide, route (experiment)	Number of rats and sex	Collection of samples during the test and at sacrifice	Duration
10 mg/kg bw, oral (single low dose)	4 males 4 females	Urine, faeces, cage wash, bile, blood, plasma, organs & tissues*, carcass	48-hours
* Comprises intestine & contents and stomach & contents			

The rats each received a single dose of [phenyl-U-14C]-fluopicolide at a target dose of 10 mg/kg bw/d (low dose). Urine, faeces and bile samples were collected at intervals until sacrifice at 48-hours, when the blood, GI tract and carcass were collected.

3. Bile cannulation surgery

Surgery was performed 24-hours prior to dosing. Male and female rats were anaesthetized with Isoflurane and the bile ducts cannulated through a mid-line incision. The cannulae were exteriorized dorsally and the body wall and sutured with surgical thread. Following surgery, the cannulae of each animal was led through the roof of the individual glass metabolism cage, permitting the collection of the bile into a weighed container. Animals showing a normal flow of bile were selected for the study.

4. Dosing

Adequate volumes of the suspension of the test compound in aqueous methyl cellulose (0.75% w/w) were administered to fasted (16-hours), cannulated rats *via* a single oral gavage dose.

5. Collection of excreta

Following administration of the radiolabelled test substance, the animals were housed in glass metabolism cages (Jencon's metabowls MK III), which permitted the separate quantitative collection of urine and faeces, for a maximum of 48-hours following dosing. Bile was individually collected into an appropriate weighed container, via each animals cannulae which was led through the roof of the metabolism cage (to avoid disturbing the rat during collection).

Urine and bile were collected at 0-6-, 6-24- and 24-48-hours following dosing, whilst faeces was collected at 24-hour intervals following dosing. Cages were washed with distilled water for each 24-hour period post-dose and then with acetonitrile at the end of the in-life phase; cage washes were retained for analysis. Samples were processed immediately if possible or were frozen until required.

6. Blood and plasma

Cardiac blood samples were taken from each animal at sacrifice, and plasma prepared by centrifugation (approximately 1000 x g for 10 minutes).

7. Sacrifice

All animals were killed by exsanguination under Imogene 500 anaesthesia, at 48-hours following dose administration.

8. Tissues and organs collected at sacrifice

The intestinal tract, intestinal tract contents, stomach and stomach contents were removed from each animal following sacrifice. The residual carcass was retained for analysis. Samples were processed immediately if possible or were frozen until required.

9. Measurement of radioactivity

Radioactivity was measured by liquid scintillation counting with automatic external standard quench correction. The limit of detection was taken to be twice the background values for blank samples in appropriate scintillation cocktails.

10. Data processing

The calculations of % dose recovery and compound concentrations (including means and standard deviations) were performed using Debra 5.2a software and Microsoft excel (some rounding up differences may have occured in the tables as an inherent feature of the software).

11. Preparation of samples for quantification of radioactivity

The following methods were used:

i) Urine, cage wash, bile, and plasma: Aliquots of liquid samples were counted in Ultima Gold liquid scintillation cocktail (10mL) after stabilizing to ambient temperature and reduced light intensity.

ii) Blood: Blood samples were combusted directly onto combusto pads contained in combusto cones.

iii) GI tract: Homogenized using an Ultra-Turrax fitted with an 8N shaft (aided by the addition of small amounts of deionised water where necessary). Portions of the homogenates were weighed into combustor-cones and combusted in a sample oxidizer following the addition of cellulose powder. The generated CO2 was trapped and mixed with a scintillation cocktail prior to radio assay.

iv) Faeces: The collected faeces were homogenized with a portion of water. Samples of various weights were combusted in a sample oxidizer following the addition of a small quantity of cellulose powder. The generated CO2 was trapped and mixed with a scintillation cocktail prior to radio assay.

II. Results and Discussion

B. Recovery

The mean recovery of radioactivity from measurements in urine, bile, and faeces, in addition to carcass and GIT at sacrifice was approximately 100.59% and 103.63% the administered radioactivity in males and females respectively. A summary of the radioactivity in percent of the administered dose found in excreta and in organs and tissues at sacrifice is presented in table 5.1.1- below.

Table 5.1.1- 22 :Recovery of radioactivity in excreta and body of rats following oral dosing of [2,6-pyridyl-14C]-fluopicolide, data expressed as % of dose administered.

Dose, route	**10 mg/kg bw**		**10 mg/kg bw**	
Experiment	**single low dose**		**single low dose**	
Duration, sex	**48 h, 4 males**		**48 h, 4 females**	
	[% dose administered]		**[% dose administered]**	
	Mean	SD	Mean	SD
Urine	5.83	2.48	10.42	5.04
Faeces	40.27	6.81	39.16	4.81
Bile	51.69	9.33	51.74	12.77
Cage wash	0.70	0.37	1.50	1.77
Tissues	2.11	1.12	0.80	0.35
Balance	**100.59**	**2.80**	**103.63**	**7.87**

B. Absorption

The estimated oral absorption, based on recoveries in urine (plus cage wash), bile and tissues (with the exclusion of the intestine and stomach contents) was 59±6.92% in males and 64.05±10.67% in females.

C. Distribution

The amount of radioactivity remaining in the total body at sacrifice, 48-hours following dosing, was relatively low and the amounts in the individual tissues were similar between the sexes, accounting for 2.11±1.12% the administered dose in male rats and 0.80±0.35% in female rats. The highest levels of radioactivity were found in the intestinal contents (1.31±0.83% in males and 0.43±0.41% in females); other tissues contained very amounts of radioactivity, ranging from <0.01% in the cardiac blood and stomach contents to 0.58% in the residual carcass.

Table 5.1.1-23 shows the radioactivity in the various organs and tissues at sacrifice expressed as µg equivalents of [2,6-pyridyl-14C]-fluopicolide.

Table 5.1.1- 23: Radioactive residues in organs and tissues at sacrifice expressed as % administered dose

Dose, route	**10 mg/kg bw**		**10 mg/kg bw**	
Experiment	**single low dose**		**single low dose**	
Duration, sex	**48 h, 4 males**		**48 h, 4 females**	
	% administered dose		**% administered dose**	
	Mean	SD	Mean	SD
Cardiac blood	0.04	0.02	0.03	0.01
Intestinal content	1.31	0.83	0.43	0.41
Intestine	0.15	0.11	0.03	0.01
Residual carcass	0.58	0.23	0.31	0.12
Cardiac plasma	0.00	0.00	0.00	0.00
Stomach	0.01	0.00	0.01	0.00
Stomach plasma	0.02	0.03	0.00	0.00
Total	**2.11**	**1.12**	**0.80**	**0.35**

D. Excretion

The rate of elimination of [2,6-pyridyl-14C]-fluopicolide was fast and similar in males and females (see table 5.1.1 below). The majority of the administered dose was excreted via the bile, followed by the faeces, with the lowest amount being found in the urine. The mean amounts of radioactivity recovered in males and females respectively were 51.69±9.33 % and 51.74±12.77 % in bile, 40.27±6.81 % and 39.16±4.81 % in faeces and 5.83±2.48 % and 10.42±5.04 % of the administered dose in urine. The total elimination of [2,6-pyridyl-14C]-fluopicolide (bile, urine, faeces, and cage wash) at 48-hours post-dose, was therefore 11.59±2.80 % in males and 103.63±7.87 % in females.

Table 5.1.1- 24: Excretion of radioactivity at time intervals, expressed as % dose administered

Dose, route	**10 mg/kg bw**		**10 mg/kg bw**	
Experiment	**single low dose**		**single low dose**	
Duration, sex	**48 h, 4 males**		**48 h, 4 females**	
Time period (h)	**[µg equivalents]**		**[µg equivalents]**	
	Mean	SD	Mean	SD
Radioactivity in faeces (% of dose administered, cumulative)				
0-24	28.19	7.53	29.06	1.76
0-48	40.27	6.81	39.16	4.81
Radioactivity in urine (% of dose administered, cumulative)				
0-6	2.11	0.72	1.88	0.52
0-24	5.20	2.38	6.95	2.77
0-48	5.83	2.48	10.42	3.04
Radioactivity in bile (% of dose administered, cumulative)				
0-6	15.94	9.27	21.37	4.63
0-24	47.08	10.05	48.91	12.46
0-48	51.69	9.32	51.74	12.77

III. Conclusions

The behaviour of [2,6-pyridyl-14C]-fluopicolide in male and female bile cannulated rats following a single oral low dose, can be characterized by the following observations:

- Oral absorption, based on recoveries in urine (plus cage wash), bile and tissues (with the exclusion of the intestine and stomach contents) was 59±6.92% in males and 64.05±10.67% in females.
- Excretion was fast and essentially complete at sacrifice (48 hours post dose). The main route of excretion was via the bile (51.69% in males and 51.74% in females), followed by the faeces (40.27% in males and 39.16% in females) and the urine (5.83% in males and 10.42% in females). Only approximately 2% and 0.8% of the administered dose remained in the body at sacrifice, in males and females respectively, reflecting the high and fast rate of excretion.
- The majority of radioactivity in the tissues at sacrifice was found in the intestinal contents of both sexes. Only a small amount of radioactivity was found in the other tissues at sacrifice, indicating that [2,6-pyridyl-14C]-fluopicolide was almost completely absorbed and/or eliminated within 48 hours post-dose.

Assessment and conclusion by applicant:

An acceptable study yielding valid conclusions

Data Point:	KCA 5.1.1/06
Report Author:	[redacted]
Report Year:	2003
Report Title:	(phenyl-U-14C)-AE C638206 and (pyridyl-2,6-14C)-AE C638206: Rat blood and plasma kinetics study
Report No:	C036987
Document No:	M-221902-01-1
Guideline(s) followed in study:	EU (=EEC): 87/302/EEC; JMAF: 59, Nohsan No 4200; USEPA (=EPA): OPPTS 870.7485
Deviations from current test guideline:	not specified
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

The pharmacokinetic behaviour of fluopicolide in the whole blood and plasma of rats was investigated with radiolabelled fluopicolide ([phenyl-U-^{14}C]-fluopicolide and [2,6-pyridyl-^{14}C]-fluopicolide).

A single oral gavage dose of radiolabelled fluopicolide (10 or 100 mg/kg bw) was administered to groups of 4 rats/sex. Achieved dose rates were 10.6 and 120.8 mg/kg for the phenyl label, and 10.3 and 106.8 mg/kg bw for the pyridyl label.

Blood samples were taken by tail snip at 0.5, 1, 2, 3, 4, 6, 8 and 24 h after dosing, then at each subsequent 24 h interval until sacrifice at 168 h.

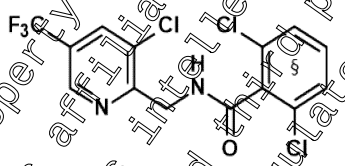
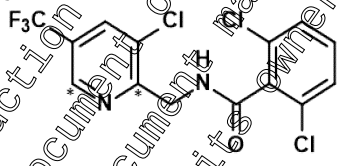
Whole blood and plasma samples were analysed by LSC.

- Maximal concentration in blood or plasma was approximately 2 µg equivalents/g after 6 to 8 h (10 mg/kg bw dose) and approximately 7 µg equivalents/g after 8 to 12 h (100 mg/kg bw dose).
- Terminal elimination half-life was approximately 100 h from whole blood.
- Total systemic exposure (as $AUC_{0\text{-inf}}$) in blood and plasma was in the region of 50 µg.h/g (10 mg/kg bw) or 300 µg.h/g (100 mg/kg bw dose).
- Exposure as demonstrated by blood and plasma kinetics was not dose-proportional, apparently owing to a lower proportion absorbed at the higher dose.

I. Materials and methods

A. Materials

1. Test Material:

IUPAC Name	2,6-dichloro-N-[[3-chloro-5-(trifluoromethyl)-2-pyridinyl]methyl]-benzamide	
CAS Name	benzamide, 2,6-dichloro-N-[[3-chloro-5-(trifluoromethyl)-2-pyridinyl]methyl]	
Code name	AE C638206	
Common name	Fluopicolide	
Empirical formula	$C_{14}H_8Cl_3F_3N_2O$	
CAS Number	239110-15-7	
Molar mass	383.59 g/mol	
Chemical structures, radiolabelled test material	[phenyl-U-^{14}C]-fluopicolide  § *Denotes uniformly labelled ring*	[2,6-pyridyl-^{14}C]-fluopicolide  * *Denotes position of label*
Lot number	CFO12747	903AE-3
Radiochemical purity	99.1% (HPLC)	>99% (HPLC)
Specific radioactivity	2148 MBq/mmol (58.05 mCi/mmol, 130.6 µCi/mg)	2298 MBq/mmol (62 mCi/mmol, 162 µCi/mg)
Non-labelled test material	Fluopicolide	
Purity	99.3%	
Lot number	R001737	
Stability of test compound	Stable at -20°C	

2. Vehicle: Aqueous methyl cellulose (0.75% w/w)

3. Test animals:

Species: Rat
Strain: Sprague Dawley, CD
Age: Not stated
Weight at dosing: 158-211 g
Source: [redacted]
Acclimation period: 6-8 days
Identification: Ear tattoo
Diet: Certified rodent diet A04C (UAR, Epinay-S/Orge, France)
Water: Provided *ad libitum* (filtered, softened water from municipal supply)
Housing: Metal wire-mesh bottomed cages in metal trays with absorbent paper during acclimatisation, then in specialised metabolism cages thereafter

Environmental conditions

Temperature:	22 ± 2˚C
Humidity:	55 ± 15%
Photoperiod:	12 hours
Air changes	Average 15 changes/hour

B. Study design and methods

1. In-life dates: 17 January to 04 March 2002

2. Dose regimen and design of test

Radiolabelled test substance	Nominal dose (mg/kg bw, single oral gavage dose)	Rats/sex	Actual mean doses (mg/kg bw)	Radioactivity content (µCi/kg)	Collection of blood samples during the test and at sacrifice
[phenyl-U-^{14}C]-fluopicolide	Low dose: 10	4/sex	Males: 10.48 Females: 10.65	133.68	0.5, 1, 2, 3, 4, 6, 8, 24 h, then at each 24 h until sacrifice at 168 h
	High dose: 100	4/sex	Male: 120.64 Female: 120.99	152.70	
[2,6-pyridyl-^{14}C]-fluopicolide	Low dose: 10	4/sex	Males: 10.32 Females: 10.36	131.32	
	High dose: 100	4/sex	Males: 106.40 Females: 107.28	132.90	

The rats each received a single dose of labelled fluopicolide at a target dose of either 10 mg/kg bw (low dose) or 100 mg/kg bw (high dose), with a nominal radioactive dose of 125 µCi/kg. Dose rate was 1 g dose suspension per 200 g body weight. Blood samples were collected at the above-stated intervals by tail snip, until sacrifice by exsanguination at 168 h.

2. Dose preparation

The doses were prepared by mixing weighed amounts of radiolabelled and non-radiolabelled material in acetonitrile to achieve a homogeneous solution at the desired specific radioactivity. The solution was then gently evaporated under a stream of nitrogen, appropriate weights of aqueous methyl cellulose (0.75% w/w) added and the resulting mixture ground with mortar and pestle to produce a suspension. The dose suspension was of sufficient quantity and concentration to provide a dose of 1 g per 200 g body weight. The dose suspensions were prepared on the day of dosing and assayed by HPLC for active ingredient content and for radioactivity content (by LSC) before, during and following the dosing procedure.

3. Dosing

Approximately 16 h prior to dosing, the diet was removed from the cages (access to water was retained), then restored within one hour after dosing. The rats were weighed immediately prior to dosing, which was a single oral dose by gavage.

4. Sample collection

The extreme tip of each animal's tail was removed with a scalpel and the blood sample was collected in a heparinized glass capillary. Samples were collected prior to dosing, then at approximately 0.5, 1, 2, 3, 4, 6, 8 and 24 h after dosing, then at each subsequent 24 h interval until and including 168 h, at which time each animal was sacrificed by exsanguination.

Plasma samples were prepared from whole-blood samples by centrifugation.

5. Quantitative analysis of radioactivity

Radioactivity was measured by liquid scintillation counting with automatic external standard quench correction. The limit of detection was taken to be twice the background values for blank samples in appropriate scintillation cocktails.

Aliquots of plasma were counted in Ultima Gold liquid scintillation cocktail (10 mL). Blood samples (up to approximately 0.2 g) were combusted directly after drying of weighed aliquots. The carbon dioxide generated was absorbed by a trapping agent, which was then mixed with Permafluor scintillation cocktail prior to radioassay.

6. Data processing

Calculations of compound concentrations (including means and standard deviations) were performed using Debra 5.2a software and Microsoft Excel (some rounding may have occurred in the tables as an inherent feature of the software). Pharmacokinetic parameters were calculated using WinNonlin 3.3.

II. Results and discussion

1. Blood and plasma kinetics: Phenyl label

Table 5.1.1- 25: Radioactivity in blood and plasma; Mean ± SD (µg [phenyl-U-^{14}C] fluopicolide equivalents/g blood)

Time (h)	10 mg/kg bw single dose				100 mg/kg bw single dose			
	Blood		Plasma		Blood		Plasma	
	Males	Females	Males	Females	Males	Females	Males	Females
0.5	306 ± 66	311 ± 70	416 ± 79	402 ± 103	1588 ± 142	1622 ± 133	1214 ± 181	1654±1071
1	632 ± 91	547 ± 109	782 ± 103	730 ± 114	1540 ± 61	1402 ± 88	1672 ± 769	2761±2406
2	776 ± 44	734 ± 92	916 ± 82	877 ± 241	2621 ± 467	2288 ± 229	3227 ± 537	2991 ± 617
3	914 ± 126	719 ± 87	1520 ± 654	866 ± 128	3246 ± 582	2938 ± 441	4304 ± 749	3423 ± 582
4	1166 ± 214	964 ± 268	1635 ± 358	1250 ± 321	4473 ± 725	3828 ± 690	5505±1169	4.666 ± 845
6	1391 ± 221	1129 ± 487	1908 ± 295	1451 ± 712	5779±1132	4675±1086	7598±1314	5796±1251
8	1490 ± 234	1148 ± 479	2199 ± 394	1527 ± 769	6731±1474	5179±1479	9224±2041	6743±1727
24	694 ± 176	713 ± 201	821 ± 195	709 ± 271	5224±1564	9516±7530	6816±2484	10363±8402
48	225 ± 44	245 ± 18	218 ± 57	82 ± 23	993 ± 724	1882 ± 641	1091 ± 348	1154±1127
72	129 ± 23	193 ± 08	88 ± 15	32 ± 2	682 ± 176	1160 ± 347	369 ± 94	248 ± 48
96	97 ± 15	170 ± 15	37 ± 8	25 ± 11	454 ± 118	950 ± 328	91 ± 106	ND
120	73 ± 12	148 ± 18	38 ± 31	34 ± 57	363 ± 131	849 ± 295	91 ± 182	ND
144	52 ± 18	126 ± 21	ND	ND	281 ± 90	731 ± 283	ND	ND
168	44 ± 10	115 ± 23	ND	ND	260 ± 79	636 ± 267	ND	ND

Table 5.1.1- 26: Pharmacokinetic parameters, Mean ± SD, [phenyl -U-^{14}C] fluopicolide

	10 mg/kg bw single dose				100 mg/kg bw single dose			
	Blood		Plasma		Blood		Plasma	
	Males	Females	Males	Females	Males	Females	Males	Females
C_{max} (µg equ/g)	1.50 ± 0.24	1.19 ± 0.44	2.20 ± 0.39	1.61 ± 0.67	7.05 ± 1.06	9.84 ± 7.25 [a]6.22 ± 0.57	9.63 ± 1.72	10.99 ± 5.92 [a]7.03 ± 0.32
T_{max} (h)	7.5 ± 1	5.5 ± 2.5	8 ± 0	6.5 ± 3	12 ± 8	20 ± 8	12 ± 8	20 ± 8
$t_{0.5}$ (h)	56.63 ± 1.61	120.7 ± 26.2	18.85 ± 1.49	19.72 ± 6.21	94.39 ± 9.20	124.7±28.5	13.7 ± 1.73	9.52 ± 1.24
$AUC_{(0-168h)}$ µg.h/g	48.04 ± 8.35	52.87 ± 8.16	54.24 ± 10.9	38.88 ± 9.84	276.8±54.7	400.5±154 [a]325.3±42.2	288.2±72.9	326.3±211.5 [a]224.1±26.0
$AUC_{(0-inf)}$ µg.h/g	51.65 ± 8.66	73.54 ± 12.6	55.22 ± 11.4	40.28 ± 8.97	311.9±59.9	521.9±146 [a]466.9±128	293.6±73.7	329.9±211 [a]224.7±26.0

a: Excluding outlier results from one female

Table 5.1.1- 27: Pharmacokinetic parameters, Mean ± SD, [2,6-^{14}C-pyridyl] fluopicolide

	10 mg/kg bw single dose				100 mg/kg bw single dose			
	Blood		Plasma		Blood		Plasma	
	Males	Females	Males	Females	Males	Females	Males	Females
C_{max} (µg equ/g)	1.49 ± 0.51	1.18 ± 0.26	2.14 ± 0.62	1.59 ± 0.23	6.34 ± 1.52	5.10 ± 0.78	9.18 ± 2.60	6.67 ± 1.38
T_{max} (h)	7 ± 1.2	6 ± 1.6	7 ± 1.2	6.5 ± 1.9	8 ± 0	8 ± 0	8 ± 0	8 ± 0
$t_{0.5}$ (h)	80.34 ± 14.3	140.3 ± 25.4	14.44 ± 2.62	12.67 ± 2.76	79.19 ± 8.12	123.8 ± 4.69	13.48 ± 3.31	9.39 ± 1.18
$AUC_{(0-168h)}$ µg.h/g	40.59 ± 13.2	45.22 ± 4.78	48.39 ± 20.3	30.61 ± 5.11	217.2 ± 28.9	244.8 ± 44.5	229.2 ± 40.1	175.3 ± 49.2
$AUC_{(0-inf)}$ µg.h/g	45.37 ± 15.2	67.72 ± 12.9	48.93 ± 20.3	30.96 ± 5.17	248.6 ± 38.2	338.6 ± 69.6	234.5 ± 40.1	180.4 ± 49.2

C_{max} = maximal concentration, T_{max} = time of maximal concentration, $t_{0.5}$ = terminal elimination half-life, AUC = area under the curve

The radiolabelled fluopicolide was absorbed moderately rapidly, with mean maximal concentrations being achieved 6 to 8 h after the low dose, 8 to 12 h after the high dose, followed by a moderately rapid elimination, such that the majority was eliminated after 48 h. There then followed a slower terminal elimination phase, with a mean half-life of approximately 100 h. The only real difference between dose levels of 10 or 100 mg/kg (nominal) was an increase in systemic exposure that was not dose-proportional (approximately 5 to 6-fold, compared with a 10 to 11-fold increase in dose). This was apparently due to a proportionately lower level of absorption at 100 mg/kg bw, as the mean C_{max} values were proportionately lower.

III. Conclusions

Following single oral gavage dosing, using either [phenyl-U-^{14}C]-fluopicolide or [2,6-^{14}C]-fluopicolide at 10 or 100 mg/kg bw in rats, the general pharmacokinetic profiles in whole blood and plasma were similar between both radiolabels and sexes:

- Maximal concentration in blood or plasma was approximately 2 µg equivalents/g after 6 to 8 h (10 mg/kg bw dose), and approximately 7 µg equivalents/g after 8 to 12 h (100 mg/kg bw dose).
- Terminal elimination half-life was approximately 100 h, from whole blood.
- Total systemic exposure (as $AUC_{0\text{-}inf}$) in blood and plasma was in the region of 50 µg.h/g (10 mg/kg bw) or 300 µg.h/g (100 mg/kg bw dose).
- Exposure as demonstrated by blood and plasma kinetics was not dose-proportional, apparently owing to a lower proportion absorbed at the higher dose.

Assessment and conclusion by applicant:
An acceptable study yielding valid results and conclusions.

Data Point:	KCA 5.1.1/07
Report Author:	[redacted]
Report Year:	2003
Report Title:	(phenyl-U-14C)-AE C638206 Rat tissue kinetic study
Report No:	C036983
Document No:	M-221892-01-1
Guideline(s) followed in study:	EU (=EEC): 87/302/EEC; JMAF: 59, Nohsan No 4200; USEPA (=EPA): OPPTS 870.7485
Deviations from current test guideline:	not specified
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

In this GLP and guideline compliant study, the tissue distribution and metabolism of [phenyl-U-^{14}C]-fluopicolide (referred to as [phenyl-U-^{14}C]- AE C638206 in the report) was investigated in male and female Sprague Dawley rats following a single oral dose at 10 and 100 mg/kg bw.

[phenyl-U-^{14}C]-fluopicolide was administered to groups of four male and female rats that were sacrificed at intervals from 8 hours to 120 hours post dose administration. Tissue distribution was determined by collecting a comprehensive range of organs and tissues and analyzing for radioactivity.

Pooled liver samples from the high dose group (at 8-hour sacrifice time) were analysed to determine the metabolites.

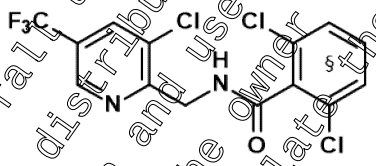
[phenyl-U-^{14}C]-fluopicolide was found to be rapidly and widely distributed into the tissues, with no notable sex difference in distribution pattern. At the high dose group proportionally less of the administered radioactivity was in the tissues compared to the low dose indicating less test substance absorption at the high dose. The highest tissue concentrations at both dose levels and over several sampling times were in the G.I tract and is likely to comprise unabsorbed material and material excreted in the bile. The next highest concentrations of radioactivity were in the liver, kidneys and adrenals with concentrations decreasing over time in these tissues.

Investigations of metabolites in liver samples revealed up to 12 different metabolites, as well as unmetabolized parent material. The 4 identified metabolites were AE C653711 (M-01), AE 0717559 (no M number assigned), AE C643890 (M-06), and AE 0717560 (no M-number assigned). All metabolites and parent material were present in the liver at levels of less than 1% of administered radioactivity.

I. Materials and methods

A. Materials

1. Test Material:

IUPAC Name	2,6-dichloro-N-[[3-chloro-5-(trifluoromethyl)-2-pyridinyl]methyl]-benzamide
CAS Name	benzamide, 2,6-dichloro-N-[[3-chloro-5-(trifluoromethyl)-2-pyridinyl]methyl]
Code name	AE C638206
Common name	Fluopicolide
Empirical formula	$C_{14}H_8Cl_3F_3N_2O$
CAS Number	239110-15-7
Molar mass	383.59 g/mol
Chemical structure	 □ *denotes position of label*
Radiolabelled test material	[Phenyl-U-^{14}C]-fluopicolide*
Lot number	CFQ12474
Radiochemical purity	99.1% (by HPLC)
Specific radioactivity	58.05 mCi/mmole or 2148 MBq/mmole
Specific radioactivity of dose suspensions	Checked by HPLC and radio assay
Stability of test compound	Stable at -20˚C

*referred to as [Phenyl-U-^{14}C]-AE C638206 in the report

2. Vehicle: Aqueous methyl cellulose (0.75% w/w)

3. Test animals:

Species:	Rat, (males and females)
Strain:	Sprague Dawley
Age:	Not stated
Weight at dosing:	163.94 – 259.06g
Source:	[redacted]
Acclimation period:	7 days
Identification:	Ear tattoo
Diet:	Certified rodent diet A04C (UAR, Villemoisson, France)
Water:	Provided *ad libitum* (filtered, softened water from municipal supply)
Housing:	Housed in metal wire-mesh bottomed cages in metal trays containing absorbent paper.
Environmental conditions	
Temperature:	22 ± 2˚C
Humidity:	55 ± 15%
Photoperiod:	12 hours
Air changes	Average 15 changes/hour

4. Preparation of dosing solutions

The doses were prepared by weighing the required amount of non-radiolabelled fluopicolide into an appropriate recipient to which a calculated volume of the radioactive solution was added to provide the required specific activity. This solution was evaporated under a gentle stream of nitrogen. The appropriate weight of vehicle was then added and the mixture ground by use of a mortar and pestle. The dose suspension was prepared 24 hours before the day of dosing and was assayed for active ingredient concentration (by HPLC) and for radioactivity content (by LSC) both before, mid-way through and after the dosing procedure.

B. Study design and methods

1. In life dates: March 20th to April 2nd, 2002

2. Dose regimen and design of tests

Rats were dosed with [phenyl-U-^{14}C]-fluopicolide as a suspension in 0.75% (w/w) aqueous methyl cellulose at a nominal single gavage dose level of 10 or 100 mg/kg bw. The dosing and animal termination plan is given in the table below.

Groups of four male and four female rats were killed at times that were calculated to correspond to the blood Cmax, Cmax/2, Cmax/4 and Cmax/10 times obtained from the pharmacokinetic study.

Table 5.1.1- 28: Study design

Group Number	N° of doses	Time of Sacrifice (hrs post dose)	N° of Rats per sacrifice time	Nominal Dose level (mg/kg bw)	Nominal ^{14}C dose level (µCi/Kg)
1 (male) 2 (female)	1	8 h males and females	4 males, 4 females	10	125
3 (male) 4 (female)		24 h males 30 h females			
5 (male) 6 (female)		36 h males 48 h females			
7 (male) 8 (female)		72 h males 120 h females			
9 (male) 10 (female)	1	8 h males & females*	4 males, 4 females	100	125
17 (male) 12 (female)		24 h males 30 h females			
13 (male) 14 (female)		48 h males 48 h females			
15 (male) 16 (female)		72 h males 120 h females			
* Pooled liver samples from this group were analysed for metabolites.					

3. Dosing

The nominal active ingredient dose levels were 10 and 100 mg/kg bw. Analytical verification of the dose checks (by HPLC) showed the mean dose rates achieved for the low dose group animals was 10.181 ± 0.126 mg/kg for the males and 10.099 ± 0.234 mg/kg for the females. The achieved mean dose rates for the high dose group animals were 100.313 ± 1.045 mg/kg for the males and 100.358 ± 0.908 mg/kg for the females.

The doses were administered via single oral gavage at a rate of 1g of dose suspension per 200 g rat body weight. The rodent diet was removed 16 hours before dosing and returned thereafter. The Group 17 (24 hour sacrifice 100 mg/kg) replaced the original group of animals (Group 11) after it was discovered that the food had not been returned within one hour after dosing.

4. Sacrifice

At each sacrifice time the rats were exsanguinated whilst under "Imalgène-500" (approx. 2 ml/kg) anaesthesia.

5. Tissues and organs collected at sacrifice

The following tissues were collected and weighed: Liver, kidney, heart, lungs, brain, spleen, pancreas, fat (abdominal), skeletal muscle, cardiac blood and plasma, eyes, ovaries (for the females), testes (for the males), intestinal tract plus contents, stomach plus contents, bone (femur) and marrow, adrenals, uterus (for the females), thyroid, harderian glands, skin & fur and any other tissues which display pathology. The residual carcass samples were also retained for analysis.

Whenever possible samples were processed, as they were collected. Remaining samples and samples awaiting processing were stored at -20°C in the dark until required.

6. Measurement of radioactivity

The amounts of radioactivity in the various samples were determined by liquid scintillation counting. Samples were counted for 10 minutes or for 2 sigma % in an appropriate scintillation cocktail using a Packard 1900 TR counter with on-line computing facilities in which quenching effects were determined using an external standard and spectral quench parameter (tSIE) method. The limit of detection was taken to be twice the background values for blank samples in appropriate scintillation cocktails.

7. Preparation of samples for quantification of radioactivity

The liver, kidney, heart, lungs, brain, spleen and muscle were homogenised using an Ultra-Turrax in appropriate volumes of HPLC-grade water (as necessary) and submitted to combustion analysis. Portions of the homogenates were weighed into Combustocones and then combusted using the sample oxidiser (Packard Model 387 Tri-Carb) following the addition of a small quantity of cellulose powder (approximately O.lg). The carbon dioxide generated by the combustion was absorbed by a trapping agent (Carbo-Sorb, 10 ml) which was then mixed with an appropriate scintillation cocktail (Permafluor, 12 ml) prior to radioassay.

The fat, testes, pancreas, bone (plus marrow) and uterus were scissor-minced and weighed directly into Combusto-cones1 before submission to combustion analysis. The adrenals, eyes, ovaries, harderian glands, thyroid and aliquots of cardiac blood (up to approximately 0.2 g) were directly combusted individually. The residual carcass, intestinal tract plus contents, stomach plus contents and skin & fur samples were solubilised in alcoholic potassium hydroxide.

8. Preparation of samples for determination of metabolic profiles

Metabolite profile was determined in samples of pooled liver extracts analysed using HPLC. The liver extracts were prepared from the livers of Groups 9 and 10 from the high dose group. These were pooled according to sex to create 2 samples for metabolite quantification and identification. Approximately 40% of the original weight of each liver homogenate was taken to prepare the pooled samples. Triplicate aliquots were assayed by combustion analysis to determine actual levels of radioactivity present in order to calculate recovery.

Pooled liver homogenates were extracted with methanol and methanol/water (1:1 v/v), partitioned with hexane and the methanolic extracts then centrifuged and concentrated under nitrogen.

8. Chromatography

The metabolite profile was investigated using a LUNA C18 HPLC column and two different gradient elution systems. The metabolites were identified by comparison with authentic standards and LC/MS/MS analyses.

High performance liquid chromatography (HPLC):

Solvent delivery:	Waters 2690 Separation module
Column:	Luna C18 (2), 250 x 4.6 mm particle size 5 µm
Injector:	Waters 2690 Separation Module
Detector:	1) Waters 2487 dual absorbance detector, wavelength 254 nm
	2) IN/US System β-RAM Model 3 radiodetector with 200 µL HPLiGl flow cell.
Mobile phase A:	Acetonitrile and 25 nM sodium acetate (pH 6.9)
Mobile phase B:	Acetonitrile + 0.5% tetrahyrofuron and 10 mM ammonium acetate + 0.1% tetrahydrofuran (pH 5.5)

Liquid chromatography / mass spectrometry (LC/MS):

The metabolite identifications were performed by using Positive and Negative Ion Electrospray/Liquid Chromatography-Mass Spectrometry/Mass Spectrometry (ESP+/-/LC-MS-MS) using a VG Quattro II mass spectrometer. The HPLC conditions corresponded to those reported in Mobile Phase B. The analysis was performed on a sample of male pooled liver.

II. Results and Discussion

A. Recovery

Radioactivity in excreta was not collected therefore total recovery was not calculated. Recovery of radioactivity following extraction in the liver homogenates yielded a total extracted recovery of 73.16% (male liver) and 83.3% (female liver). Total recovery (including post extraction solids) was 97.64% (male liver) and 99.83% (female liver).

Before radio-HPLC analysis the methanolic extract was concentrated under a stream of nitrogen and re-dissolved in methanol. The total recovery in the methanolic extracts following concentration was 61.17% and 67.81% for the males and female liver respectively.

B. Tissue distribution

The following tables present the mean concentrations of radioactivity found in the tissues at the sampling times following single oral doses of [phenyl-U-^{14}C]-AE C638206 at the nominal rates of 10 and 100 mg/kg bw.

Table 5.1.1- 29: Evolution of Radioactivity Levels in the Tissues of Male Rats Following a Single Oral Dose of [phenyl-U-^{14}C]-fluopicolide at the rate of 10 mg/kg b/w, (results expressed as µg [Phenyl-U-^{14}C]-fluopicolide equivalents per g tissue)

Sample	Males									
	8h post dose		24h post dose		36h post dose		72h post dose		168h post dose*	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Adrenal	5.169	1.088	1.417	0.516	1.031	0.198	0.554	0.253	0.140	0.023
Bone & Marrow	0.595	0.153	0.229	0.023	0.133	0.019	0.040	0.009	0.021	0.004
Brain	0.503	0.104	0.277	0.038	0.174	0.039	0.069	0.007	0.027	0.003
Carcass	1.044	0.068	0.452	0.044	0.299	0.050	0.135	0.010	0.098	0.001
Cardiac blood	2.263	0.375	0.743	0.156	0.424	0.064	0.183	0.025	0.120	0.010
Cardiac Plasma	3.466	0.481	0.896	0.234	0.428	0.095	0.087	0.024	0.019	0.006
Eyes	0.533	0.077	0.262	0.030	0.145	0.041	0.051	0.009	0.017	0.002
Fat	3.730	0.526	0.339	0.074	0.168	0.037	0.058	0.010	0.029	0.006
Harder's Gland	1.534	0.434	0.912	0.119	0.663	0.101	0.399	0.088	0.151	0.020
Heart	1.539	0.254	0.629	0.052	0.436	0.053	0.249	0.010	0.149	0.012
Intestine & contents	53.709	8.824	10.723	1.573	4.951	1.241	0.721	0.345	0.075	0.037
Kidneys	4.207	0.523	2.430	0.314	1.673	0.163	0.802	0.132	0.314	0.036
Liver	5.931	0.387	2.926	0.338	2.385	0.453	0.992	0.200	0.429	0.039
Lungs	1.689	0.213	0.556	0.071	0.331	0.038	0.130	0.011	0.056	0.004
Muscle	0.771	0.140	0.382	0.075	0.263	0.043	0.135	0.008	0.092	0.005
Pancreas	1.381	0.195	0.541	0.099	0.309	0.043	0.131	0.016	0.078	0.008
Skin & Fur	1.298	0.144	0.471	0.082	0.410	0.094	0.126	0.012	0.095	0.027
Spleen	1.181	0.208	0.438	0.085	0.247	0.034	0.097	0.011	0.043	0.007
Stomach & contents	0.748	0.820	0.407	0.295	0.157	0.082	0.040	0.008	0.016	0.010
Testes	1.132	0.490	0.371	0.048	0.214	0.043	0.071	0.006	0.025	0.003
Thyroids	1.940	0.450	0.713	0.127	0.384	0.067	0.115	0.078	ND	NA

* data from previous ADME study: see [redacted] 2001; M-204781-01-1
ND: not detected (below twice background).
NA: not applicable.
SD: Standard deviation

Table 5.1.1- 30: Evolution of Radioactivity Levels in the Tissues of Female Rats Following a Single Oral Dose of [phenyl-U-^{14}C]-fluopicolide at the rate of 10 mg/kg bw (results expressed as µg [Phenyl-U-^{14}C]-fluopicolide equivalents per g tissue)

Sample	Females									
	8h post dose		30h post dose		48h post dose		120h post dose		168h post dose*	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Adrenal	5.370	0.777	0.988	0.195	0.534	0.061	0.199	0.018	0.156	0.041
Bone & Marrow	0.477	0.089	0.103	0.015	0.054	0.008	0.026	0.009	0.019	0.001
Brain	0.317	0.054	0.086	0.017	0.053	0.006	0.030	0.003	0.023	0.002
Carcass	0.972	0.077	0.228	0.024	0.154	0.015	0.100	0.013	0.086	0.005
Cardiac blood	1.655	0.519	0.482	0.034	0.302	0.037	0.214	0.037	0.183	0.026
Cardiac Plasma	2.329	0.883	0.290	0.065	0.094	0.009	0.022	0.005	0.015	0.005
Eyes	0.315	0.052	0.082	0.016	0.047	0.009	0.016	0.003	0.014	0.003
Fat	10.855	3.261	0.347	0.019	0.194	0.037	0.041	0.016	0.050	0.005
Harder's Gland	1.438	0.276	0.433	0.075	0.373	0.049	0.181	0.009	0.117	0.017
Heart	1.266	0.181	0.374	0.031	0.251	0.026	0.172	0.023	0.129	0.005
Intestine & contents	69.311	9.611	12.009	3.249	1.835	0.678	2.932	5.252	0.036	0.008
Kidneys	4.722	0.494	1.385	0.108	0.807	0.025	0.390	0.062	0.253	0.056
Liver	4.883	0.225	1.880	0.293	1.067	0.073	0.499	0.033	0.305	0.038
Lungs	1.512	0.285	0.334	0.041	0.186	0.021	0.088	0.016	0.075	0.009
Muscle	0.542	0.131	0.208	0.025	0.145	0.010	0.093	0.011	0.076	0.008
Ovaries	2.468	0.755	0.305	0.042	0.161	0.012	0.050	0.018	0.044	0.007
Pancreas	1.189	0.232	0.262	0.021	0.168	0.019	0.081	0.012	0.071	0.020
Skin & Fur	1.871	0.428	0.448	0.139	0.280	0.038	0.133	0.015	0.080	0.006
Spleen	1.013	0.163	0.242	0.022	0.146	0.020	0.086	0.021	0.066	0.010
Stomach & contents	6.701	11.985	0.222	0.114	0.133	0.118	0.064	0.037	0.031	0.010
Thyroids	3.252	2.628	0.398	0.056	0.208	0.035	ND	NA	ND	NA
Uterus	2.765	2.672	0.272	0.082	0.129	0.059	0.026	0.006	0.016	0.001

* data from previous ADME study: see [redacted] 2000; M-204781-01-1
ND: not detected (below twice background)
NA: not applicable
SD: Standard deviation.

Table 5.1.1- 31: Evolution of Radioactivity Levels in the Tissues of Male Rats Following a Single Oral Dose of [phenyl-U-^{14}C]- fluopicolide at the rate of 100 mg/kg bw (results expressed as µg [Phenyl-U-^{14}C]- fluopicolide equivalents per g tissue)

Samples	Males									
	8h post dose		24h post dose		48h post dose		72h post dose		168h post dose*	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Adrenal	14.32	1.75	6.39	0.87	2.96	0.96	1.37	0.32	0.47	0.09
Bone & Marrow	2.11	0.33	1.17	0.15	0.44	0.12	0.22	0.07	ND	NA
Brain	2.50	0.40	1.59	0.30	0.62	0.15	0.34	0.10	0.09	0.02
Carcass	4.07	0.60	2.08	0.34	0.79	0.13	0.55	0.13	0.51	0.19
Cardiac blood	6.45	1.50	3.65	0.84	1.39	0.27	0.82	0.25	0.39	0.15
Cardiac Plasma	9.68	2.28	4.37	0.20	1.20	0.43	0.53	0.20	0.02	0.05
Eyes	2.11	0.57	1.50	0.31	0.56	0.14	0.28	0.11	0.02	0.03
Fat	22.03	6.31	2.20	0.43	0.58	0.08	0.24	0.08	0.13	0.16
Harder's Gland	7.17	1.28	3.04	0.34	1.68	0.27	1.15	0.24	0.49	0.06
Heart	4.91	0.87	2.78	0.45	1.25	0.19	0.81	0.15	0.40	0.04
Intestine & contents	594.42	72.95	63.97	17.13	12.44	5.21	3.02	2.04	0.18	0.17
Kidneys	13.30	1.89	9.29	1.21	4.92	1.33	2.71	0.59	0.88	0.06
Liver	17.69	2.27	10.35	1.91	6.13	1.20	3.48	0.91	1.15	0.06
Lungs	5.38	0.94	2.93	0.48	1.16	0.18	0.63	0.21	0.21	0.10
Muscle	3.21	0.71	1.76	0.22	0.78	0.17	0.47	0.11	0.19	0.02
Pancreas	6.71	2.58	3.35	1.10	1.12	0.36	0.54	0.16	0.18	0.12
Skin & Fur	9.06	7.32	2.63	0.75	1.11	0.22	0.75	0.20	0.51	0.05
Spleen	3.67	0.62	2.55	0.43	0.97	0.19	0.53	0.23	0.07	0.14
Stomach & contents	14.02	1.93	1.77	0.44	1.08	0.34	0.59	0.16	0.07	0.05
Testes	3.46	0.55	1.95	0.30	0.75	0.19	0.42	0.15	0.06	0.04
Thyroids	5.90	0.57	2.90	0.68	1.13	0.77	ND	NA	ND	NA

* data from previous ADME study; see [redacted] (2001; M-204781-01-1)
ND: not detected (below twice background).
NA: not applicable.
SD: Standard deviation.

Table 5.1.1- 32: Evolution of Radioactivity Levels in the Tissues of Female Rats Following a Single Oral Dose of [phenyl-U-^{14}C]- fluopicolide at the rate of 100 mg/kg bw (results expressed as µg [Phenyl-U-^{14}C]- fluopicolide equivalents per g tissue)

Sample	Female									
	8h post dose		30h post dose		48h post dose		120h post dose		168h post dose*	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Adrenal	18.13	0.86	5.32	0.77	3.26	0.42	0.89	0.03	0.71	0.08
Bone & Marrow	2.05	0.37	0.77	0.13	0.38	0.10	0.11	0.03	ND	NA
Brain	2.72	0.26	0.69	0.05	0.48	0.07	0.12	0.00	0.09	0.06
Carcass	6.57	1.37	1.38	0.27	0.97	0.16	0.43	0.04	0.55	0.05
Cardiac blood	5.14	0.94	3.95	0.72	2.04	0.59	1.10	0.23	0.60	0.09
Cardiac Plasma	6.80	1.73	4.00	1.05	0.85	0.32	0.12	0.01	0.06	0.04
Eyes	1.21	0.49	0.78	0.08	0.41	0.09	0.09	0.01	0.08	0.01
Fat	59.41	7.36	2.72	0.65	1.21	0.21	0.25	0.05	0.20	0.05
Harder's Gland	11.08	1.19	2.81	0.27	1.83	0.10	0.87	0.06	0.69	0.04
Heart	4.90	0.29	2.23	0.28	1.40	0.28	0.61	0.04	0.70	0.09
Intestine & contents	843.16	167.71	97.26	27.84	12.59	3.70	0.54	0.22	0.14	0.04
Kidneys	17.61	1.42	8.58	0.92	4.75	0.88	1.77	0.15	1.38	0.17
Liver	18.24	2.79	10.09	0.92	5.99	1.36	2.06	0.13	1.75	0.12
Lungs	5.78	0.67	2.49	0.35	1.45	0.25	0.48	0.07	0.30	0.07
Muscle	3.75	0.34	1.34	0.37	0.82	0.21	0.33	0.03	0.38	0.07
Ovaries	14.24	2.59	2.60	0.39	1.23	0.16	0.19	0.13	0.22	0.02
Pancreas	10.41	6.07	2.03	0.48	1.11	0.23	0.33	0.03	0.42	0.13
Skin & Fur	10.67	2.51	2.46	0.31	1.64	0.20	0.76	0.02	1.56	0.68
Spleen	4.70	1.27	1.78	0.52	1.16	0.21	0.45	0.06	0.19	0.13
Stomach & contents	94.95	88.65	2.62	2.30	2.09	1.50	0.22	0.07	0.20	0.04
Thyroids	6.61	0.49	2.36	0.17	1.19	0.80	ND	NA	ND	NA
Uterus	9.06	3.46	2.42	0.54	0.88	0.20	0.14	0.01	0.07	0.09

* data from previous ADME study: see [redacted] 2001; M-204781-01-1
ND: not detected (below twice background).
NA: not applicable.
SD: Standard deviation.

Low dose: 10 mg/kg bw

Low dose males

At eight hours post dose the highest mean concentrations of radioactivity in male tissues were found in the intestine and contents (53.71 µg equiv./g) followed by the liver (5.93 µg equiv./g), adrenals (5.17 µg equiv./g), kidneys (4.21 µg equiv./g), fat (3.73 µg equiv./g), plasma (3.47 µg equiv./g) and blood (2.26 µg equiv./g). The thyroids, lungs, heart, harder's gland, pancreas, skin & fur, spleen, testes and carcass all presented mean concentrations that were between 1 and 2 µg equiv./g. The muscle, stomach and contents, bone & marrow, eyes and brain presented mean concentrations between 0.5 and 1 µg equiv./g.

Thereafter the mean concentrations of radioactivity found in each of the tissues from the males decreased at each sampling time. Between the 8 and the 24 hour sampling time the mean concentrations decreased by *ca* 91% in the fat (to 0.339 µg equiv./g), 80% in the intestine and contents (to 10.72 µg equiv./g) and between 41% (harder's gland) to 74% (plasma) in all the other tissues.

Levels in the tissues from the males at 168 hours post dose had decreased by a mean of 95.2 ± 3.5% from the concentrations observed at 8 hours post dose. The highest mean concentrations of radioactivity in male tissues at 168 hours post dose were found in the liver (0.43 µg equiv./g) and the kidneys (0.31 µg equiv./g). The heart, harder's gland, adrenals and blood presented mean concentrations that were between 0.12 and 0.15 µg equiv./g. The remaining tissues possessed concentrations that were <0.1 µg equiv./g.

Low dose females

At eight hours post dose the highest mean concentrations of radioactivity in female tissues were found in the intestine and contents (69.31 µg equiv./g) followed by the fat (10.86 µg equiv./g), stomach & contents (6.70 µg equiv./g), adrenals (5.37 µg equiv./g), liver (4.88 µg equiv./g), kidneys (4.72 µg equiv./g) and thyroids (3.25 µg equiv./g). The uterus, ovaries and plasma were found to possess levels between 2.33 and 2.77 µg equiv./g. The lungs, heart, harder's gland, pancreas, skin & fur, spleen, blood and carcass all presented mean concentrations that were between 0.97 and 1.87 µg equiv./g. The muscle, bone & marrow, eyes and brain presented mean concentrations between 0.32 and 0.54 µg equiv./g.

Thereafter the mean concentrations of radioactivity found in each of the tissues from the females decreased at each sampling time with the exception of the 120 hour intestine and contents which increased from 1.84 µg equiv./g observed at 48 hours to 2.93 µg equiv./g and the levels in fat at 168 hour post dose which increased compared to those observed at 120 hours post dose (0.04 to 0.05 µg equiv./g). However, examination of the variability, using the standard deviation, shows that both apparent increases where not significant. Between the 8 and the 30 hour sampling time the mean concentrations decreased by *ca* 97% in the fat (to 0.34 µg equiv./g) and the stomach and contents (to 0.22 µg equiv./g). Mean concentrations fell by 87 to 90% in the uterus (to 0.27 µg equiv./g), the thyroids (to 0.40 µg equiv./g), the ovaries (to 0.31 µg equiv./g) and the plasma (to 0.29 µg equiv./g). Levels fell by between 61% (liver) to 83% (intestine and contents) in all the other tissues.

Levels in the tissues from the females at 168 hours post dose had decreased by a mean of 95.1 ± 3.9% from the concentrations observed at 8 hours post dose. The highest mean concentrations of radioactivity in female tissues at 168 hours post dose were found in the liver (0.31 µg equiv./g) and the kidneys (0.25 µg equiv./g). The heart, harder's gland, adrenals and blood presented mean concentrations that were between 0.12 and 0.18 µg equiv./g. The remaining tissues possessed concentrations that were <0.1 µg equiv./g.

Comparison of low dose males and females

Comparison of the results obtained for the males and the females at the 8 hour time point showed that there was very little difference between the two sexes with the mean concentrations of radioactivity observed in the majority of the tissues not being significantly different. One exception to this was the fat where the mean concentration found in the samples from the males was 3.73 ± 0.53 µg equiv./g and the mean concentration observed in the samples from the females was 10.86 ± 3.26 µg equiv./g. There was a small difference in the mean concentrations observed in the livers from males (5.93 ± 0.39 µg equiv./g) and the females (4.88 ± 0.23 µg equiv./g).

The next time points were 24 hours for the males and 30 hours for the females. Given the similarity of the results at 8 hours it is, perhaps, not surprising that the mean concentrations observed in the majority of the samples from the females were lower than the corresponding male tissues as they were obtained 8 hours later. Exceptions to this were the fat (males: 0.34 ± 0.07 µg equiv./g, females: 0.35 ± 0.02 µg equiv./g) and the intestine and contents (males: 10.72 ± 1.57 µg equiv./g, females: 12.01 ± 3.25 µg equiv./g) where the concentrations were similar.

The differences observed between the 24 h and 30 h time points continued to be observed in the next set of sampling times of 36 hours for the males and 48 hour for the females. Twenty of the 22 sampled tissues displayed differences in radioactivity concentrations with the higher levels being present in the male tissues. The two tissues that contained similar concentrations were the fat (0.17 ± 0.04 µg equiv./g and 0.19 ± 0.04 µg equiv./g for males and females respectively) and the stomach and contents (0.16 ± 0.08 µg equiv./g and 0.13 ± 0.12 µg equiv./g for males and females respectively).

A similar picture was seen when comparing the 72 hour male results with the 120 hour female results as, again, the concentrations in the tissues from the females were either lower or equivalent to those observed in the male tissues.

At the 168 hour time point the mean concentrations observed in the tissues were the same in both sexes with the only exceptions of the whole blood, liver and spleen. The concentrations in the blood and spleen were only slightly higher in the females (0.18 ± 0.03 and 0.07 ± 0.01 µg equiv./g respectively) compared to the males (0.12 ± 0.01 and 0.04 ± 0.01 µg equiv./g respectively). In the liver the mean concentrations were found to be slightly higher for the males (0.43 ± 0.04 µg equiv./g) compared to the females (0.31 ± 0.04 µg equiv./g).

High dose: 100 mg/kg bw

High dose males

At eight hours post dose the highest mean concentrations of radioactivity in male tissues were found in the intestine and contents (594.42 µg equiv./g) followed by the fat (22.03 µg equiv./g), liver (17.69 µg equiv./g), adrenals (14.32 µg equiv./g), stomach and contents (14.02 µg equiv./g) and kidneys (13.30 µg equiv./g). Remaining tissues all presented mean concentrations that were <10 µg equiv./g. The lowest mean concentrations at 8 hours were observed in the bone & marrow and the eyes at 2.11 µg equiv./g.

Thereafter the mean concentrations of radioactivity found in each of the tissues from the males decreased at each sampling time. Between the 8 and the 24 hour sampling time the mean concentrations decreased by *ca* 90% in the fat (to 2.21 µg equiv./g), 89% in the intestine and contents (to 63.97 µg equiv./g) and 87% in the stomach and contents (to 1.77 µg equiv./g). Decreases in mean concentrations between 8 and 24 hours post dose in the other tissues ranged from 29% for the eyes to 71% for the skin & fur.

Levels in the tissues from the males at 168 hours post dose had decreased by a mean of 96.3 ± 3.4% from the concentrations observed at 8 hours post dose. The highest mean concentrations of radioactivity in male tissues at 168 hours post dose were found in the liver (1.15 µg equiv./g) and the kidneys (0.88 µg equiv./g). The heart, harder's gland, adrenals, skin and fur, carcass and blood presented mean concentrations that were between 0.39 and 0.51 µg equiv./g. The remaining tissues possessed concentrations that were ≤0.21 µg equiv./g.

High dose females

At eight hours post dose the highest mean concentrations of radioactivity in female tissues were found in the intestine and contents (843.16 µg equiv./g) followed by the stomach & contents (94.93 µg equiv./g), fat (59.41 µg equiv./g), liver (18.24 µg equiv./g), adrenals (18.13 µg equiv./g) and kidneys (17.61 µg equiv./g). The ovaries, harder's gland, pancreas and skin & fur were found to possess levels between 10.17 and 14.24 µg equiv./g. the remaining tissues all presented mean concentrations that were <10 µg equiv./g. The lowest mean concentrations at 8 hours were observed in the bone & marrow and the eyes at 2.05 µg equiv./g and 1.21 µg equiv./g respectively.

Thereafter the mean concentrations of radioactivity found in each of the tissues from the females decreased at each sampling time up until 120 hours post dose. Between 120 and 168 hours tissues either displayed a decrease or no significant difference due to high variability. Between the 8 and the 30 hour sampling time the mean concentrations decreased by *ca* 97% in the stomach and contents (to 2.62 µg equiv./g), and 95% in the fat (to 2.72 µg equiv./g). Mean concentrations fell by 81 to 89% in the pancreas (to 2.03 µg equiv./g), the ovaries (to 2.60 µg equiv./g), and the intestine and contents (to 97.26 µg equiv./g). Levels fell by between 23% (blood) to 79% (carcass) in all the other tissues.

Levels in the tissues from the females at 168 hours post dose had decreased by a mean of 94.8 ± 4.8% from the concentrations observed at 8 hours post dose. The highest mean concentrations of radioactivity in female tissues at 168 hours post dose were found in the liver (1.75 µg equiv./g), the kidneys (1.38 µg equiv./g) and the skin & fur (1.56 µg equiv./g). The adrenals, heart, harder's gland, blood, carcass, pancreas, muscle, lungs, ovaries, stomach and contents, fat, spleen and intestine and contents presented mean concentrations that were between 0.14 and 0.71 µg equiv./g. The remaining tissues possessed concentrations that were <0.1 µg equiv./g.

Comparison of high dose males and females

As seen at the lower dose level, there was very little difference in the mean tissue concentrations between the two sexes at the 8 hour time point. Where there were observable differences, the tissue concentrations observed in the females were found to be higher than those observed in the males. At the 8 hour time point this was observed for the adrenals, carcass, fat, harder's gland, intestine and contents and the kidneys. The tissue where there was the biggest difference was the fat where the mean concentration found in the samples from the males was 22.03 ± 6.31 µg equiv./g and the mean concentration observed in the samples from the females was 59.41 ± 7.36 µg equiv./g. The difference in levels in the adrenals, carcass, harder's gland, and kidneys ranged between 2.5 and 4.3 µg equiv./g whilst the difference between the intestine and contents was much greater associated with a much greater variability in the results.

The next time points were 24 hours for the males and 30 hours for the females. Sixteen out of the 22 sampled tissues were found to contain similar concentrations between the sexes. None of the exceptions to this demonstrated differences that were greater than 1 µg equiv./g.

At 48 hours post dose (both sexes) the similarities were more striking as 18 out of the 22 sampled tissues possessed the same levels of radioactivity with only the fat (0.58 ± 0.08 and 1.21 ± 0.21 µg equiv./g for males and females respectively) and the skin & fur (1.11 ± 0.22 and 1.64 ± 0.20 µg equiv./g for males and females respectively) exhibiting any differences (albeit small) between the sexes.

The larger gap between sampling times of 72 hours for the males and 120 hours for the females appeared to accentuate the differences with only 10 of the sampled tissues possessing the same levels in both sexes. However, the largest of the differences was only *ca* 2.5 µg equiv./g which was observed in the intestine and contents (higher levels in males). The mean difference between the sexes for the remaining tissues was only *ca* 0.5 µg equiv./g.

At the 168 hour time point the mean concentrations observed in the tissues were the same in both sexes for 11 out of the 22 tissues sampled. Once again, those tissues that presented differences in mean concentrations between the sexes displayed only relatively small differences ranging between 0.06 µg equiv./g (eyes) to 1.05 µg equiv./g (skin & fur). In all of these cases the females were found to possess the slightly higher concentrations.

Comparison of low and high dose tissue distribution

Comparison of the ratio of the concentrations observed in the tissues from the males following a single oral administration at a nominal 10 and 100 mg/kg b/w reveals that the increases in tissue concentrations in the high dose group were not proportional to the increase in dose rate. The mean increase in tissue concentrations was of the order of x3.8 (range x2.8 to x7.0) excluding the stomach and contents and intestine and contents. Initial differences in concentrations in the GIT samples were approximately dose proportional and then descended to similar values to those seen in the other tissues.

Comparison of the ratio of the concentrations observed in the tissues from the females following a single oral administration at a nominal 10 and 100 mg/kg b/w reveals that the increases in tissue concentrations in the high dose group were not proportional to the increase in dose rate. The mean increase in tissue concentrations was of the order of x5.8 (range x2 to x9.1) excluding the stomach and contents, intestine and contents, plasma at 30 hours post dose (x13.8) and the skin and fur at 168 hours post dose (x19.4). Initial differences in concentrations in the GIT samples were approximately dose proportional and then descended to similar values to those seen in the other tissues albeit only at 120 hours post dose for the stomach and contents.

These results imply that the [phenyl-U-^{14}C]-fluopicolide was proportionately less well absorbed at the higher dose level of 100 mg/kg compared to the lower dose level of 10 mg/kg. These results are in agreement with those reported from the blood and plasma kinetic study (see [redacted] 2002, M-221902-01-1).

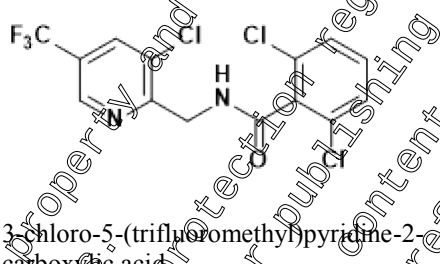
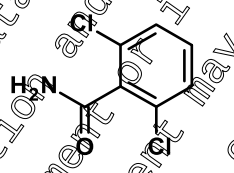
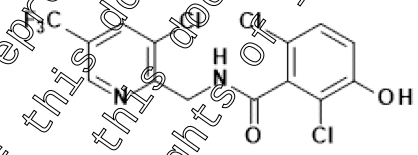
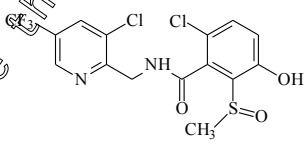
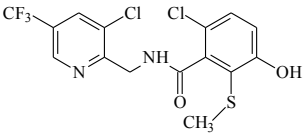
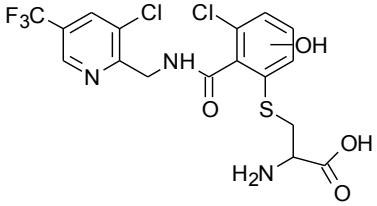
C. Metabolism

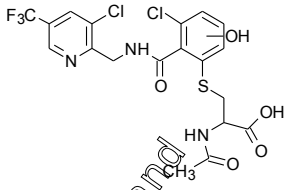
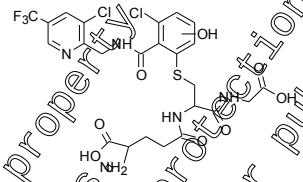
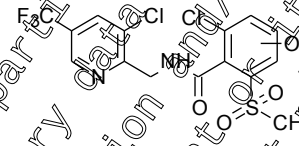
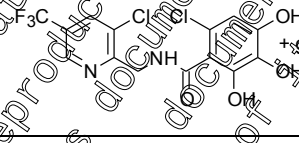
The HPLC system using Mobile Phase A and Mobile Phase B compared the retention times of the peaks from the concentrated liver extract with the retention times of the standards. Mobile Phase B gave improved resolution of peaks and was used for the LC/MS/MS investigations with a sample of male liver extract.

Using HPLC a total of 13 radioactive fractions were observed with 12 in the male extracts and 11 in the female extracts. The radioactive fractions present in the liver extracts were found to represent between <0.01 to 0.20% of the administered radioactivity. Mass spectroscopic investigations permitted the identification of five of the radioactive fractions by comparison with authentic standards. Three additional structures were proposed based on LC/MS/MS investigations.

The metabolites identified in the liver are shown in the table below:

Table 5.1.1- 33: Metabolites identified in the liver following a single oral dose of [phenyl-U-14C]-fluopicolide

Metabolite Code	% administered dose	Chemical Structure and name (IUPAC)
Fluopicolide (AE C638206, BCS-AM59797)	0.04% and 0.20% dose in males and females respectively	 3-chloro-5-(trifluoromethyl)pyridine-2-carboxylic acid
M-01 (BAM, AE C653711, BCS-AA65784)	0.09% and 0.08% dose in males and females respectively	 2,6-dichlorobenzamide
M-06 (AE C643890 BCS-AW63671)	0.03% and 0.10% dose in males and females respectively	 2,6-dichloro-N-{[3-chloro-5-(trifluoromethyl)pyridin-2-yl]methyl}-3-hydroxybenzamide
AE 0717559	0.02% and 0.01% dose in males and females respectively	
AE 0717560	0.08% and 0.09% dose in males and females respectively	
Fraction N° 6 (similar to M-25) Proposed to have an uncleaved structure where the dichlorophenyl ring had been hydroxylated on either the 3, 4 or 5 position and the chlorine atom at the 2 position had been replaced by glutathione conjugation and subsequent biotransformation of the glutathione to leave cysteine.		

Fraction 7 Similar to fraction number 6 but with the addition of an N-acetylation of the cysteine conjugate.		
Proposed structure for metabolite not detected by radio-HPLC	Below 0.01% of adminsitered dose.	
Proposed structure for metabolite not detected by radio-HPLC	Below 0.01% of adminsitered dose.	
Proposed structure for metabolite not detected by radio-HPLC	Below 0.01% of adminsitered dose.	

Investigations into the metabolites present in pooled liver extracts from males and females sacrificed at 8 hours post dose at 100 mg/kg b/w indicated that the metabolic profiles were qualitatively similar between the sexes with differences only being observed where metabolites were present at very low levels in one sex and not observed in the other, suggesting that the difference may have been related to the sensitivity of the chromatography.

III. Conclusions

In conclusion [phenyl-U-^{14}C]-fluopicolide administered at a single oral dose of 10 and 100 mg/kg bw to male and female rats can be characterized by the following observations:

- [phenyl-U-^{14}C]-fluopicolide was found to be rapidly and widely distributed into the tissues.
- There did not appear to be a significant sex difference.
- The difference in achieved concentrations in the tissues between the two dose levels was not dose proportional indicating that proportionately less of the administered radioactivity was absorbed at the higher dose rate.
- The highest tissue concentrations were observed in the intestine and contents in both sexes and both dose levels over several sampling times. This is probably a reflection of a combination of unabsorbed material and biliary excretion for the early samples and continued biliary excretion for later time points as was demonstrated in the bile excretion study ([redacted] 2003; M-230976-01-1, and [redacted] 2002; M-212243-01-1).
- The next highest concentrations were consistently observed in the liver, kidneys and adrenals albeit that the concentrations were decreasing with time post dosing.
- Investigations into the metabolites present in the liver at 8 hours post dosing revealed that [phenyl-U-^{14}C]-fluopicolide was already extensively metabolised with up to 15 different radioactive fractions being observed.
- Five of these fractions were identified as AE C653711 (0.09% and 0.08% dose in males and females respectively), AE 0717559 (0.02% and 0.01% dose), AE C643890 (0.03% and 0.10% dose), AE 0717560 (0.08% and 0.09% dose) and AE C638206 (0.04% and 0.20% dose in males and females respectively).

Assessment and conclusion by applicant:

The study is valid and acceptable to investigate the tissue distribution and metabolism of [phenyl-U-^{14}C]-fluopicolide.

Data Point:	KCA 5.1.1/08
Report Author:	[redacted]
Report Year:	2003
Report Title:	(2,6-pyridyl-14C)-AE C638206 - Rat tissue kinetic study
Report No:	C036980
Document No:	M-221885-01-1
Guideline(s) followed in study:	EU (=EEC): 94/79/EC; JMAF: 12, Nousan No 8147; USEPA (=EPA): OPPTS 870.7485
Deviations from current test guideline:	not specified
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

In this GLP and guideline compliant study, the tissue distribution and metabolism of [2,6-pyridyl-^{14}C]-fluopicolide (referred to as [2,6-pyridyl-^{14}C]- AE C638206 in the report) was investigated in male and female Sprague Dawley rats following a single oral dose at 10 mg/kg bw.

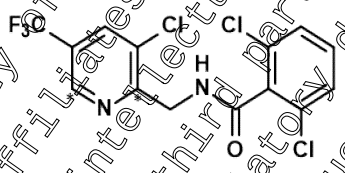
[2,6-pyridyl-^{14}C]-fluopicolide was administered to groups of four male and female rats that were sacrificed at intervals from 7 to 48 hours post dose in males, and 6 to 120 hours post dose administration in females. Tissue distribution was determined by collecting a comprehensive range of organs and tissues and analyzing for radioactivity.

[2,6-pyridyl-^{14}C]-fluopicolide was found to be rapidly and widely distributed into the tissues, with no notable sex difference in distribution pattern. The highest tissue concentrations at both dose levels and over several sampling times were in the G.I tract and is likely to comprise unabsorbed material and material excreted in the bile. The next highest concentrations of radioactivity were in the liver, kidneys and adrenals and cardiac blood with concentrations decreasing over time in these tissues.

I. Materials and methods

A. Materials

1. Test Material:

IUPAC Name	2,6-dichloro-N-[[3-chloro-5-(trifluoromethyl)-2-pyridinyl]methyl]-benzamide
CAS Name	benzamide, 2,6-dichloro-N-[[3-chloro-5-(trifluoromethyl)-2-pyridinyl]methyl]
Code name	AE C638206
Common name	Fluopicolide
Empirical formula	$C_{14}H_8Cl_3F_3N_2O$
CAS Number	239110-15-7
Molar mass	383.59 g/mol
Chemical structure	 * denotes position of label
Radiolabelled test material	[2,6-pyridyl-^{14}C]-fluopicolide*
Lot number	GAR2034/4
Radiochemical purity	97.26 % (by HPLC)
Specific radioactivity	70.61 mCi/mmole or 2612.6 MBq/mmole
Specific radioactivity of dose suspensions	Checked by HPLC and radio assay
Stability of test compound	Stable at -20°C

*referred to as [Pyridyl-2,6-^{14}C]-AE C638206 in the report

2. Vehicle: Aqueous methyl cellulose (0.75% w/w)

3. Test animals:

Species: Rat, (males and females)
Strain: Sprague Dawley CD strain
Age: Not stated
Weight at dosing: 176.74 – 229.25g
Source: [redacted]
Acclimation period: 7 days
Identification: Ear tattoo
Diet: Certified rodent diet A04C (UAR, Villemoisson, France)
Water: Provided *ad libitum* (filtered, softened water from municipal supply)
Housing: Housed in metal wire-mesh bottomed cages in metal trays containing absorbent paper.

Environmental conditions
Temperature: 22 ± 2˚C
Humidity: 55 ± 15%
Photoperiod: 12 hours
Air changes: Average 15 changes/hour

4. Preparation of dosing solutions

The doses were prepared by weighing the required amount of non-radiolabelled fluopicolide into an appropriate recipient to which a calculated volume of the radioactive solution was added to provide the required specific activity. This solution was evaporated under a gentle stream of nitrogen. The appropriate weight of vehicle was then added and the mixture ground by use of a mortar and pestle. The dose suspension was prepared 24 hours before the day of dosing and was assayed for active ingredient concentration (by HPLC) and for radioactivity content (by LSC) both before, mid-way through and after the dosing procedure.

B. Study design and methods

1. In life dates: November 13th, 2002 to January 03rd 2003

2. Dose regimen and design of tests

Rats were dosed with [pyridyl-2,6-^{14}C]-fluopicolide as a suspension in 0.75% (w/w) aqueous methyl cellulose at a nominal dose level of 10 mg/kg bw. The dosing and animal termination plan is given in the table below.

Groups of four male and four female rats were killed at sequential times following dose administration that were calculated to correspond to the blood Cmax, Cmax/2, Cmax/4 and Cmax/10 times obtained from the pharmacokinetic study.

Table 5.1.1- 34: Study design

Group Number	N° of doses	Time of Sacrifice (hrs post dose)	N° of Rats per sacrifice time	Nominal Dose level (mg/kg bw)	Nominal ^{14}C dose level (µCi/Kg)
1 (male) 2 (female)	1	7 h males and 6h females	4 males, 4 females	10	125
3 (male) 4 (female)		24 h males 24 h females			
5 (male) 6 (female)		36 h males 36 h females			
7 (male) 8 (female)		48 h males 120 h females			

3. Dosing

The nominal active ingredient dose level was 10 mg/kg bw. Analytical verification of the dose checks (by HPLC) showed the mean dose rates achieved for the low dose group animals was 10.19 ± 0.16 mg/kg for the males and 10.21 ± 0.17 mg/kg for the females.

The doses were administered via single oral gavage at a rate of 1g of dose suspension per 200 g rat body weight. The rodent diet was removed 16 hours before dosing and returned thereafter.

4. Sacrifice

At each sacrifice time the rats were exsanguinated whilst under "Imalgène-500" (approx. 2 ml/kg) anaesthesia.

5. Tissues and organs collected at sacrifice

The following tissues were collected and weighed: Liver, kidney, heart, lungs, brain, spleen, pancreas, fat (abdominal), skeletal muscle, cardiac blood and plasma, eyes, ovaries (for the females), testes (for the males), intestinal tract plus contents, stomach plus contents, bone (femur) and marrow, adrenals, uterus (for the females), thyroid, harderian glands, skin & fur and any other tissues which display pathology. The residual carcass samples were also retained for analysis.

Whenever possible samples were processed, as they were collected. Remaining samples and samples awaiting processing were stored at -20°C in the dark until required.

6. Measurement of radioactivity

The amounts of radioactivity in the various samples were determined by liquid scintillation counting. Samples were counted for 10 minutes or for 2 sigma % in an appropriate scintillation cocktail using a Packard 1900 TR counter with on-line computing facilities in which quenching effects were determined using an external standard and spectral quench parameter (tSIE) method. The limit of detection was taken to be twice the background values for blank samples in appropriate scintillation cocktails.

7. Preparation of samples for quantification of radioactivity

Plasma was allowed to stabilise to ambient temperature before being counted using 10 mL Ultima Gold liquid scintillation cocktail.
The liver, kidney, heart, lungs, brain, spleen and muscle were homogenised using an Ultra-Turrax in appropriate volumes of HPLC-grade water (as necessary) and submitted to combustion analysis. Portions of the homogenates were weighed into Combustocones and then combusted using the sample oxidiser (Packard Model 387 Tri-Carb) following the addition of a small quantity of cellulose powder (approximately 0.lg). The carbon dioxide generated by the combustion was absorbed by a trapping agent (Carbo-Sorb, 10 ml) which was then mixed with an appropriate scintillation cocktail (Permafluor, 12 ml) prior to radioassay.
The testes and bone (plus marrow) were scissor-minced and weighed directly into Combusto-cones1 before submission to combustion analysis. The adrenals, eyes, ovaries, harderian glands, thyroid, uterus, pancreas and aliquots of cardiac blood (up to approximately 0.2 g) were solubilised in 1 mL Soluene before addition of Hionic Fluor scintillation cocktail. Between 100 to 300 mg of fat were solubilised in Soluene/isopropanol (1:1 v/v). The residual carcass, intestinal tract plus contents, stomach plus contents, muscle skin & fur samples were solubilised in alcoholic potassium hydroxide.

II. Results and Discussion

A. Recovery

Radioactivity in excreta was not collected therefore total recovery was not calculated. The actual mean achieved dose rates for both sexes was 10.2 mg/kg bw.

B. Tissue distribution

The following tables present the mean concentrations of radioactivity found in the tissues at the sampling times following single oral doses of [2,6-pyridyl-^{14}C]-fluopicolide at the nominal rates of 10 mg/kg bw.

Table 5.1.1- 35: Evolution of Radioactivity Levels in the Tissues of Male Rats Following a Single Oral Dose of [pyridyl-2,6-^{14}C]-fluopicolide at the rate of 10 mg/kg bw (results expressed as µg [Pyridyl-2,6-^{14}C]- fluopicolide equivalents per g tissue)

Sample	Males									
	7 hours		24 hours		36 hours		48 hours		168 hours*	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Adrenal	5.400	0.121	0.667	0.099	0.451	0.122	0.217	0.068	0.057	0.059
Bone & Marrow	0.418	0.080	0.084	0.007	0.057	0.015	0.028	0.005	0.023	0.015
Brain	0.211	0.039	0.053	0.018	0.033	0.006	0.021	0.004	0.002	0.004
Carcass	0.717	0.464	0.099	0.012	0.134	0.013	0.066	0.012	0.022	0.010
Cardiac blood	1.091	0.317	0.437	0.028	0.331	0.070	0.211	0.041	0.158	0.054
Cardiac Plasma	1.625	0.520	0.465	0.037	0.313	0.123	0.109	0.041	0.040	0.035
Eyes	0.178	0.035	0.065	0.016	0.036	0.009	0.020	0.004	0.002	0.004
Fat	5.843	0.943	0.272	0.066	0.153	0.045	0.087	0.017	0.010	0.009
Harder's Gland	1.205	0.185	0.164	0.016	0.117	0.033	0.057	0.011	0.018	0.010
Heart	0.924	0.231	0.233	0.054	0.164	0.028	0.075	0.023	0.043	0.017
Intestine & contents	41.521	8.617	4.932	1.461	2.912	0.395	1.126	0.568	0.230	0.266
Kidneys	2.812	0.413	0.792	0.196	0.733	0.202	0.329	0.082	0.115	0.062
Liver	4.601	0.799	1.983	0.309	1.652	0.414	0.719	0.140	0.166	0.059
Lungs	1.286	0.369	0.266	0.033	0.192	0.042	0.094	0.024	0.050	0.024
Muscle	0.439	0.143	0.143	0.011	0.060	0.015	0.037	0.009	0.009	0.004
Pancreas	2.316	1.500	0.189	0.058	0.109	0.022	0.091	0.029	0.014	0.014
Skin & Fur	0.535	0.132	0.231	0.028	0.163	0.027	0.144	0.017	0.041	0.015
Spleen	0.686	0.168	0.179	0.020	0.129	0.023	0.071	0.012	0.048	0.019
Stomach &contents	5.944	6.047	0.095	0.039	0.075	0.018	0.024	0.004	0.009	0.005
Testes	0.465	0.110	0.113	0.014	0.068	0.014	0.030	0.007	0.010	0.006
Thyroids	1.432	0.419	0.253	0.026	0.229	0.070	0.095	0.025	ND	NA

*data from previous ADME study, [redacted]; 2001; M-202609-02-1
ND: not detected (below twice background).
NA: not applicable.
SD: Standard deviation

Table 5.1.1- 36: Evolution of Radioactivity Levels in the Tissues of Female Rats Following a Single Oral Dose of [pyridyl-2,6-^{14}C]-fluopicolide at the rate of 10 mg/kg bw (results expressed as µg [Pyridyl-2,6-^{14}C]-fluopicolide equivalents per g tissue)

Sample	Females									
	6 hours		24 hours		36 hours		120 hours		168 hours*	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Adrenal	5.821	0.517	1.001	0.130	0.604	0.059	0.067	0.007	0.063	0.012
Bone & Marrow	0.427	0.045	0.111	0.033	0.055	0.012	0.013	0.002	0.015	0.005
Brain	0.216	0.061	0.048	0.012	0.032	0.008	0.005	0.004	0.006	0.002
Carcass	0.517	0.091	0.109	0.038	0.176	0.066	0.040	0.006	0.024	0.004
Cardiac blood	0.945	0.194	0.621	0.028	0.412	0.042	0.314	0.116	0.296	0.024
Cardiac Plasma	1.353	0.285	0.444	0.110	0.185	0.035	0.012	0.008	0.027	0.021
Eyes	0.194	0.025	0.063	0.017	0.039	0.007	0.000	0.000	0.003	0.004
Fat	12.097	1.474	0.578	0.054	0.300	0.104	0.059	0.015	0.029	0.008
Harder's Gland	1.365	0.325	0.232	0.034	0.135	0.017	0.026	0.004	0.024	0.009
Heart	1.042	0.171	0.300	0.123	0.178	0.028	0.062	0.030	0.057	0.021
Intestine & contents	58.616	9.338	7.983	2.733	3.780	1.347	0.056	0.004	0.027	0.002
Kidneys	4.184	0.318	1.083	0.203	0.779	0.150	0.164	0.016	0.115	0.006
Liver	4.378	0.472	2.071	0.396	1.475	0.382	0.196	0.021	0.134	0.004
Lungs	1.176	0.142	0.376	0.061	0.289	0.054	0.075	0.009	0.092	0.011
Muscle	0.494	0.049	0.142	0.020	0.070	0.024	0.024	0.004	0.011	0.002
Ovaries	2.882	0.536	0.333	0.079	0.179	0.026	0.028	0.005	0.028	0.006
Pancreas	2.880	2.670	0.340	0.116	0.158	0.026	0.031	0.004	0.026	0.005
Skin & Fur Total	1.536	0.373	0.344	0.029	0.247	0.025	0.197	0.046	0.048	0.003
Spleen	0.821	0.138	0.237	0.068	0.200	0.012	0.088	0.007	0.107	0.008
Stomach & contents	0.614	0.340	0.174	0.099	0.084	0.016	0.034	0.009	0.012	0.006
Thyroids	1.228	0.527	0.376	0.023	0.232	0.047	ND	NA	ND	NA
Uterus	1.713	0.876	0.465	0.188	0.169	0.041	0.019	0.003	0.016	0.005

* data from previous ADME study, see [redacted] 2001; M-202609-02-1
ND: not detected (below twice background).
NA: not applicable.
SD: Standard deviation.

Males

At seven hours post dose the highest mean concentrations of radioactivity in male tissues were found in the intestine and contents (41.52 µg equiv./g) followed by the stomach & contents (5.94 µg equiv./g), fat (5.84 µg equiv./g), adrenals (5.40 µg equiv./g), liver (4.60 µg equiv./g), kidney (2.81 µg equiv./g), and pancreas (2.32 µg equiv./g). The plasma, blood, thyroids, lungs and Harder's gland presented mean concentrations that were between 1 and 1.63 µg equiv./g. The heart, carcass, spleen, skin & fur, testes, muscle, bone & marrow, brain and eyes presented mean concentrations between 0.92 and 0.18 µg equiv./g.

Thereafter the mean concentrations of radioactivity found in each of the tissues from the males decreased at each sampling time with the exception of the carcass at the 36 hour sampling time. Between the 7 and the 24 hour sampling time the mean concentrations decreased by *ca* 98% in the stomach & contents (to 0.1µg equiv./g), 95% in the fat (to 0.27 µg equiv./g), 92% in the pancreas (to 0.19 µg equiv./g), 88% in the intestine and contents (to 4.93 µg equiv./g) and the adrenals (to 0.67 µg equiv./g). The decreases in mean concentrations in the remaining tissues ranged from *ca* 86% (carcass & Harder's gland) to 56% (liver and skin & fur).

Levels in the tissues from the males at 168 hours post dose had decreased by a mean of 96.8 ± 1.4% from the concentrations observed at 7 hours post dose. The highest mean concentrations of radioactivity in male tissues at 168 hours post dose were found in the intestine & contents (0.23 µg equiv./g), the liver (0.17 µg equiv./g), the blood (0.16 µg equiv./g) and the kidneys (0.12 µg equiv./g). The remaining tissues possessed concentrations that were <0.06 µg equiv./g.

Females

At six hours post dose the highest mean concentrations of radioactivity in female tissues were found in the intestine and contents (58.62 µg equiv./g) followed by the fat (12.10 µg equiv./g), adrenals (5.82 µg equiv./g), liver (4.38 µg equiv./g) and kidneys (4.18 µg equiv./g). The uterus, ovaries, pancreas, skin & fur, harder's gland, thyroids, lungs, heart and plasma were found to possess mean levels between 2.88 and 1.04 µg equiv./g. The remaining tissues presented mean concentrations between 0.95 (blood) and 0.19 (eyes) µg equiv./g.

Thereafter the mean concentrations of radioactivity found in each of the tissues from the females decreased at each sampling time with the exception of the 36 hour residual carcass which increased from 0.109 µg equiv./g observed at 24 hours to 0.176 µg equiv./g and the bone & marrow, brain, spleen, lungs, and plasma levels at 168 hours post dose compared to 120 hours post dose. However examination of the means and the variability (in terms of standard deviation) indicates that the increases in the means would not be considered as being biologically or experimentally significant. Between the 6 and the 24 hour sampling time the mean concentrations decreased by *ca* 95% in the fat (to 0.56 µg equiv./g). Mean concentrations fell by ca 88% in the ovaries (to 0.33 µg equiv./g) and the pancreas (to 0.34 µg equiv./g). Mean concentrations fell by *ca* 86 to 78% in the intestine & contents (to 7.98 µg equiv./g), the harder's gland (to 0.23 µg equiv./g), the adrenals (to 1.00 µg equiv./g), the carcass (to 0.11 µg equiv./g), the brain (to 0.05 µg equiv./g) and the skin & fur (to 0.34 µg equiv./g). Levels fell by between 74% (kidneys) to 34% (blood) in all the other tissues.

Levels in the tissues from the females at 168 hours post dose had decreased by a mean of 95.9 ± 6.7% from the concentrations observed at 6 hours post dose. The highest mean concentrations of radioactivity in female tissues at 168 hours post dose were found in the blood (0.30 µg equiv./g), the liver (0.13 µg equiv./g), the kidneys (0.12 µg equiv./g) and the spleen (0.11 µg equiv./g). The lungs, adrenals, heart, skin & fur, fat, ovaries, plasma, intestine & contents, pancreas, carcass and Harder's gland presented mean concentrations that were between 0.09 and 0.02 µg equiv./g. The remaining tissues possessed concentrations that were ≤0.016 µg equiv./g.

Comparison of males and females

Comparison of the results obtained for the males and the females at the 7 and 6 hour time points showed that there was very little difference between the two sexes with the mean concentrations of radioactivity observed in the majority of the tissues being similar. Exceptions to this were the fat where the mean concentration found in the samples from the males was 5.84 ± 0.94 µg equiv./g and the mean concentration observed in the samples from the females was 12 ± 1.47 µg equiv./g, the kidney (males, 2.81 ± 0.41 µg equiv./g and females 4.18 ± 0.32 µg equiv./g) and the skin & fur (males, 0.54 ± 0.13 µg equiv./g and females 1.54 ± 0.37 µg equiv./g).

The next time points were 24 hours for both sexes. Generally, the distribution of the radioactivity was similar between the sexes. The mean concentration of radioactivity in the tissues from the males at 24 hours post dose was 0.56 ± 1.09 µg equiv./g whilst the mean concentration of radioactivity in the tissues from the females was 0.79 ± 1.67 µg equiv./g. The mean concentrations in seven of the samples from the females were higher than the corresponding male tissues (adrenals, blood, fat, Harder's gland, lungs, skin & fur and the thyroid). The remaining tissues (except the testes, ovaries and uterus) possessed similar concentrations.

At 36 hours the majority of the tissues continued to present similar mean concentrations of radioactivity between the sexes. The mean concentration of radioactivity in the tissues from the males at 36 hours post dose was 0.39 ± 0.68 µg equiv./g whilst the mean concentration of radioactivity in the tissues from the females was 0.44 ± 0.81 µg equiv./g. Only the skin & fur (males, 0.16 ± 0.02 µg equiv./g and females 0.25 ± 0.03 µg equiv./g)and the spleen (males, 0.13 ± 0.02 µg equiv./g and females 0.20 ± 0.01 µg equiv./g) presented significant differences where the mean concentrations were higher in the female tissues.

Comparing the 48-hour male results with the 120-hour female results, perhaps not too surprisingly, reveals that the majority of the tissue concentrations were lower in the female tissues than the male tissues. Equivalent concentrations were observed however in the blood (supporting the previous blood kinetic study, M-221902-01-1 Fisher and Vinck, 2003) the fat, heart, lung, muscle, spleen, stomach & contents and the skin & fur. The mean concentration of radioactivity in the tissues from the males at 48 hours post dose was 0.17 ± 0.27 µg equiv./g whilst the mean concentration of radioactivity in the tissues from the females at 120 hours post dose was 0.07 ± 0.08 µg equiv./g.

At the 168-hour time point the mean concentrations observed in the tissues were essentially the same in both sexes with the only exceptions of the whole blood, fat, lungs and spleen. In all four cases the concentrations were higher in the females. The mean concentration of radioactivity in the blood was 0.16 ± 0.05 and 0.30 ± 0.02 µg equiv./g in the males and females respectively, that in the fat was 0.01 ± 0.01 µg equiv./g (males) and 0.029 ± 0.01 µg equiv./g females. The mean concentration of radioactivity in the lungs was 0.05 ± 0.03 and 0.09 ± 0.01 µg equiv./g in the males and females respectively, that in the spleen was 0.05 ± 0.02 µg equiv./g (males) and 0.11 ± 0.01 µg equiv./g females. The mean concentration of radioactivity in the tissues from the males at 168 hours post dose was 0.05 ± 0.06 µg equiv./g whilst the mean concentration of radioactivity in the tissues from the females was 0.05 ± 0.07 µg equiv./g.

The highest tissue concentrations were observed in the intestine and contents in both sexes over all the sampling times for the males and up to 36 hours post dose for the females. This is probably a reflection of a combination of unabsorbed material and biliary excretion for the early samples and continued biliary excretion for later time points as was demonstrated in the bile excretion study (see M-230976-01-1, Gutierrez 2003).

III. Conclusions

In conclusion [pyridyl-2,6-^{14}C]-fluopicolide administered at a single oral dose of 10 bw to male and female rats can be characterized by the following observations:

- [pyridyl-2,6-^{14}C]-fluopicolide was found to be rapidly and widely distributed into the tissues.
- Thereafter a significant and rapid decrease in tissue concentrations of (approximately 96% decrease) between 6/7 hours post dose and 168 hours post dose.
- There did not appear to be a significant sex difference.
- The highest radioactivity concentrations were observed in the intestine & contents at all sampling times for the males and up to 36 hours post dose for the females and probably reflects the presence of biliary excretion of radioactivity.
- The next highest concentrations were consistently observed in the liver, kidneys, adrenals and cardiac blood albeit that the concentrations were decreasing with time post dosing.

Assessment and conclusion by applicant:

The study is valid and acceptable to investigate the tissue distribution and metabolism of [2,6-pyridyl-^{14}C]-fluopicolide.

Data Point:	IIA 5.1.1/09
Report Author:	[redacted]
Report Year:	2004
Report Title:	[Pyridyl-2,6-14C]-AE C638206: Rat metabolism following administration of a single oral low dose
Report No:	C039580
Document No:	M-227023-02-1
Guideline(s) followed in study:	EU (=EEC): 87/307/EEC, JMAF, 12, Nousan No. 8147, NOV 24, 2000, 59 Nousan 4200; USEPA (=EPA): OPPTS 870.7485
Deviations from current test guideline:	not specified
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

The metabolism of fluopicolide (reported as AE C638206) was investigated in rats with radiolabelled fluopicolide ([Pyridyl-2,6-^{14}C]-fluopicolide).

Male and female rats (4/sex) received a single oral low dose (10 mg/kg bw) of [Pyridyl-2,6-^{14}C]-fluopicolide. Animals were kept for 7 days following dosing during which time the excreta were collected and quantified and the metabolic profile and identification examined by HPLC and mass spectrometry.

Up to 28 radioactive components in the urine and up to 31 radioactive components in the faecal extracts were detected. Thus, it appeared that [Pyridyl-2,6-^{14}C]-fluopicolide was extensively metabolised in the rat following oral dosing.

In total 92.88% of the radioactivity eliminated in the urine by the male rats has been assigned a proposed structure that accounted for 16.19% of the administered dose. For the females a total of 82.6% of the radioactivity eliminated in the urine by the female rats has been assigned a proposed structure which accounted for 17.10% of the administered dose.

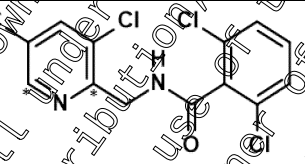
A total of 51.46% of the administered radioactivity which had been eliminated via the faeces by the male rats was assigned a structure. Similarly, a total of 55.88% of the administered radioactivity which had been eliminated via the faeces by the female rats was assigned a structure.

By summing the components that were assigned structures in the urine and the faeces, a total of 67.64% of the administered radioactivity has been assigned a structure for the males and 72.99% of the administered radioactivity for the females.

I. Materials and methods

A. Materials

1. Test Material:

IUPAC Name	2,6-dichloro-N-[(3-chloro-5-(trifluoromethyl)-2-pyridinyl]methyl]-benzamide
CAS Name	Benzamide, 2,6-dichloro-N-[[3-chloro-5-(trifluoromethyl)-2-pyridinyl]methyl]
Code name	AE C638206
Common name	Fluopicolide
Empirical formula	$C_{14}H_8Cl_3F_3N_2O$
CAS Number	239110-15-7
Molar mass	383.59 g/mol
Chemical structure	 *Denotes position of label
Radiolabelled test material	[2,6-pyridyl-^{14}C]-fluopicolide[1]
Lot number	903AE-3
Radiochemical purity	>99% (HPLC)
Specific radioactivity	2.298 GBq/mmole (62.0 mCi/mmole)
Specific radioactivity of dose suspensions	Checked by HPLC and radio assay
Stability of test compound	Stable at -20C

[1]: referred to as [2,6-pyridyl-^{14}C]-AE C638206 in the report

2. Vehicle: 0.75% (w/v) aqueous methyl cellulose

3. Test animals:

Species: Rat
Strain: Sprague Dawley CD
Age: Not staed
Weight at dosing: 168-187 g
Source: [redacted]
Acclimation period: Not stated
Identification: Ear tattoo
Diet: Certified rodent diet M20 (Pietrement, Provins, France)

Water:	Provided *ad libitum* (filtered, softened water from municipal supply)
Housing:	Housed in metal wire-mesh bottomed cages in metal trays containing absorbent paper during acclimatisation and in specialised metabolism cages thereafter
Environmental conditions	
Temperature:	22 ± 2˚C
Humidity:	55 ± 15%
Photoperiod:	12 hours
Air changes:	Average 15 changes/hour

4. Preparation of dosing solutions

The doses were prepared by mixing weighed amounts of radiolabelled and non-radiolabelled material in acetonitrile to achieve a homogenous solution at the desired specific radioactivity. The solution was then gently evaporated under a stream of nitrogen, appropriate weights of aqueous methyl cellulose (0.75% w/w) added and the resulting solution ground to produce a suspension. The dose suspension was of sufficient quantity and concentration to provide a dose of 5 g per kg rat body weight. The dose suspensions were prepared on the day of dosing and assayed by HPLC for active ingredient content and for radioactivity content (by LSC) before, during and following the dosing procedure.

B. Study design and methods

1. In life dates: 20 November 2000 – 18 June 2001

2. Dose regimen and design of tests

Table 5.1.1- 37: Study design

Administered single dose of [2,6-pyridyl-^{14}C]-fluopicolide, route (experiment)	Number of rats and sex	Collection of samples during the test and at sacrifice	Duration
10 mg/kg bw, oral (single low dose)	4 males, 4 females	Urine, faeces, cage wash, blood, plasma, organs, tissues, carcass	7 days

The rats each received a single dose of [2,6-pyridyl-U-^{14}C]-fluopicolide at a target dose of 10 mg/kg bw/d (low dose). Urine and faeces samples were collected at intervals until sacrifice at 168-hours, when the blood, organs, tissues and carcass were collected.

3. Dosing

Adequate volumes of the suspension of the test compound in aqueous methyl cellulose (0.75% w/w) were administered to fasted (18-hours) rats via a single oral gavage dose at a nominal low dose of 10 mg/kg bw. The doses for each individual animal were calculated based on the weights of the animal prior to dosing (food was replaced one-hour post dose and remained available ad libitum throughout the remainder of the study).

4. Collection of excreta

Following administration of the radiolabelled test substance, the animals were kept in specialized individual metabolism units (Jencon's metabowls MK III), which permitted the separate quantitative collection of urine and faeces, for a maximum of 168-hours following dosing. Both urine and faeces were collected frozen over solid CO_2 in weighed polystyrene boxes; carded cotton protected the samples from light.

Urine was collected at 0-6 and 6-24 hours and subsequently at 24-hour intervals following dosing whilst faeces was collected at 24-hour intervals following dosing. Cages were washed with distilled water for each 24-hour period post-dose and then with acetonitrile at the end of the in-life phase; cage washes were retained for analysis. The radioactivity content was determined by HPLC.

The faeces and urine samples contained almost all of the recovered radioactivity. Thus the metabolism study was performed with these two categories of samples. Based on the quantity of radioactivity excreted in these samples, it was decided to study time intervals of 0-6h, 6-24h, 24-48h and 48-72h for male and female urine samples and time intervals of 0-24h, 24-48h, 48-72h for male faecal samples and 0-24h and 24-48h for female faecal samples.

Because no animal presented levels of recovered radioactivity which differed significantly from the mean of the same sex, it was assumed that no qualitative difference had occurred between animals of the same sex. Thus, it was decided to study the metabolism for selected time collections by using pooled samples for males and females.

5. Preparation of Pooled Samples

Pooled Urine Samples

Several sets of pooled urine were prepared during the course of the study. Pool A was used for metabolite quantification, pool B was used for mass spectrometry analysis, pool C was used to for further metabolite identification work and a fourth pool was prepared for hydrolysis experiments.

Pool A and B

Samples were pooled according to sex and time period. Pooled samples for the male and female groups: 0-6 hours, 6-24 hours, 24-48 hours and 48-72h were prepared for method development and metabolite identification by mass spectrometry analysis. The radioactivity content of the pooled samples was determined. The samples were centrifuged for 20 minutes at 2000 G, 4°C. The supernatants were taken, aliquoted and counted. The pellets were extracted with 10 to 15 mL of acetonitrile overnight under shaking. These samples were centrifuged, and the acetonitrile phase was retained, aliquoted, counted and added to the supernatant. The samples were concentrated under a gentle stream of nitrogen to reach an approximate radioactivity level which would allow 100 000 dpm to be injected within a maximum of *ca* 200 µL.

Pool C

Samples were pooled according to sex and time period. The pooled samples for the male and female groups: 0-6 hours, 6-24 hours, 24-48 hours and 48-72h were prepared for metabolite quantification. The Pools for the time points 24-48 hours and 48-72 hours were concentrated by evaporation under a gentle stream of nitrogen. Aliquots were weighed and counted, and the final radioactive content was determined by liquid scintillation counting prior to HPLC analysis and ranging between *ca* 1.55 x 10^6 and 0.165 x 10^6 dpm/g.

Pool for hydrolysis experiments

Pooled urine samples for the hydrolysis study were prepared similarly to pool A and B except that no centrifugation was performed prior incubation. Briefly, samples were pooled according to sex and time period. The following pooled samples for the male and female groups: 0-6 hours, 6-24 hours and 24-48 hours were created according to the percentage of the recovered radioactivity detected in urine (between 20 and 55%, depending on the sample). The samples were concentrated under a nitrogen stream to reach an approximate radioactivity level of *ca* 3.3 to 4.8 x 10^6 dpm within 2.5 mL, depending on the sample.

Pooled Faeces Samples

Phase One

As for the urine, samples were pooled according to sex and time period to create pooled samples for metabolite quantification and identification. The following pooled samples 0-24 hours, 24-48 hours and 48-72h for the male group and 0-24h and 24-48h for the female group were created according to the percentage of the recovered radioactivity detected in faeces (5 or 10%, depending on the sample). The homogenate pooled samples were centrifuged at 2500 G for 30 min and the aqueous supernatants retained. The pellet was sequentially extracted with:

1. acetonitrile (twice);
2. with an acetonitrile/water mixture (70/30, v/v);
3. with chloroform;
4. with a mixture of acetonitrile/water (80/20, v/v) adjusted to pH 11 with NH_3;
5. with a mixture of acetone/water (60/40, v/v) adjusted to pH 11 with NH_3;
6. with a mixture of acetonitrile/water (60/40, v/v) adjusted to pH 4 with acetic acid;
7. with a mixture of acetone/water (60/40, v/v) adjusted to pH 11 with NH_3;
8. with a mixture of ethyl acetate/hexane/tetrahydrofuran/toluene (25/25/25/25 by volume);
9. and finally with methanol.

Each extraction was performed with ca 25-30 mL of solvent, with 15 minutes of sonication and a minimum of 4 hours under shaking conditions. Supernatants and pellets were separated after centrifugation (2500 G, 30 minutes). The radioactivity content of the supernatants was determined prior their being pooled together and concentrated under a stream of nitrogen.

Prior to injection for HPLC analysis the samples presented three phases upon centrifugation. The upper layer was visually noted as being "fatty" in nature (probably containing some residual hexane). The middle layer was a mixture of water and acetonitrile and the lower layer was made of solid residues. It was decided to further process these samples to allow HPLC analysis to be performed. The further work is described under the heading of Phase Two.

Pellets from Phase One were further extracted with a mixture of methanol/water (50/50, v/v) with 0.1M HCl for 6 hours with shaking and then with a mixture of methanol/water (50/50, v/v) with 2M HCl with shaking for 48 hours. After centrifugation (4000 G, 20 min) the supernatants were retained, and aliquots counted. The supernatants designated as "acidic extracts" were pooled with the previous supernatants. They were evaporated to dryness under nitrogen and re-dissolved in 1 mL of a mixture of acetonitrile/ water (70/30, v/v). Upon dissolution and centrifugation (20 minutes, 4000 G), two phases occurred. Both upper and lower phases were analysed using the HPLC method.

Phase Two

The extracts produced in Phase One were not suitable for HPLC analysis as they contained three distinct layers following centrifugation The middle layer was taken and referred to as ACN1.

The remaining content (top layer) was mixed and extracted with *ca* 5 mL of hexane with shaking conditions for 15 minutes. Following centrifugation, (10 minutes at 4000 G), the hexane phase was retained. This step was repeated with *ca* 2 mL hexane and the hexane phases pooled together. The resulting hexane phase was concentrated under nitrogen to *ca* 1 mL. This phase was extracted with acetonitrile (*ca* 4 mL) three times for 20 minutes with shaking.

Centrifugation (4000 G for 10 minutes) aided the separation of the hexane and acetonitrile phases. Along the last extraction, the remaining hexane phase was evaporated completely. The resulting acetonitrile phases were pooled together, concentrated under a stream of nitrogen to *ca* 0.5-1 mL and referred to as ACN2.

The remaining phase (solid residue) was resuspended in a mixture of acetonitrile/hexane and extracted on three occasions under shaking conditions. Aliquots from the first and last extraction (acetonitrile phase) were counted and it was shown that the radioactivity from the last extraction was negligible. Upon centrifugation the acetonitrile phase and the hexane phase were separated and added to their corresponding phases of the previous step (ACN2).

6. Metabolites Separation and Quantification

The quantification of the metabolites present in the samples of urine and faeces was performed using the HPLC systems described as follows:

High Performance Liquid Chromatography (HPLC) of Urine

Solvent delivery:	Waters 2690 Alliance Separation Module System.
Injections:	Chamber maintained at 4°C, injection volumes up to 100 µL.
Detectors:	l) Waters 996 Photodiode Array (λ = 254 nm) 2) IN/US System β-RAM Radiodetector, Lablogic (400ul LiGl cell, serial number 11184G).
Column:	Waters X-Terra C18, 250 x 4.6 mm, particle size 5µm Metabolism Column N°: CMET00/18
Data processor:	IBM-compatible personal computer equipped with the Laura Measuring/Evaluation software.
HPLC System:	
Mobile phase:	A = Ammonium acetate buffer (1 mM) in Distilled Water v/v) B = Acetonitrile

Time (min)	%A	%B	Gradient curve
Initial	95	5	*
10	95	5	6
30	75	25	6
60	75	25	6
65	45	55	6
75	45	55	6
85	5	95	6
90	5	95	6
92	95	5	6
110	95	5	6

Flow rate: 0.6 mL/min

High Performance Liquid Chromatography (HPLC) for analysis of faeces

Solvent delivery: Waters 2690 Alliance Separation Module System.
Column: X-Terra C18, 250 x 4.6 mm (Interchim), particle size 5µm
Metabolism Column N°.: CMET00/18

HPLC System:
Mobile phase: A = Distilled Water/TFA (0.05% v/v).
B = Acetonitrile

Time (min)	%A	%B	Gradient curve
Initial	75	25	*
10	75	25	6
30	65	35	6
45	60	40	6
55	55	45	6
65	50	50	6
75	40	60	6
80	5	95	6
81	75	25	6
95	75	25	6

Flow rate: 1.0 mL/min
Injector: Alliance, Waters with refrigerated sample compartment maintained at 4°C.
Injection volumes: Up to 100 µL
Detector: 1) Waters 996 Photodiode Array (λ = 215 nm)
2) IN/US System β-RAM Radiodetector, Lablogic (400uL LiGl cell, serial number 11184).
Data Processor: IBM-compatible personal computer equipped with the Laura Measuring/Evaluation software.

High Performance Liquid Chromatography (HPLC) for analysis of acidic extracts of faeces.

Solvent delivery: Waters 2690 Alliance Separation Module System.
Column: X-Terra C18, 250 x 4.6 mm (Interchim), particle size 5µm
Metabolism Column N°.: CMET99/06

HPLC System:
Mobile phase: A = Acetonitrile/THF (70/30, v/v)
B = Distilled water

Time (min)	%A	%B	Gradient curve
Initial	50	50	*
10	50	50	6
25	95	5	6
35	95	5	6
36	50	50	6
50	50	50	6

Flow rate: 1.0 mL/min
Injector: Alliance, Waters 2690 Separation Module System.
Injection volumes: Up to 130 µL
Detector: IN/US System β-RAM Model 3 Radiodetector (200uL LiGl).
Data Processor: Compaq Pentium II computer equipped with the Laura (VI.4a) Measuring/Evaluation software.

7. Metabolite Characterisation and Identification

Enzymatic Hydrolysis of Urine Samples

Aliquots of pooled urine from males and females (0-6 hours, 6-24 hours, 24 -48 hours) were added to glass vials containing 0.2 M sodium acetate buffer (0.2M, pH 5) to which either p- Glucuronidase (type Bl from Bovine liver) or sulphatase (type HI from Helix Pomatia) was added. The experimental protocol is described in the following table.

	Control C1 (µL)	Control C2 (µL	Sulfatase hydrolysis S1 (µL)	Sulfatase hydrolysis + Saccharolactone S2 (µL)	Glucuronidase hydrolysis S3 (µ/L)
Pooled Urine Sample	400	400	400	400	400
Glucuronidase	-	-	-		300
Sulfatase	-	-	300	300	
Saccharolactone	-	100		100	-
Buffer	400	300	100		100

The glucuronidase solution (5000 U/mL) was prepared by dissolving 0.018 g in acetate buffer (6 mL). The sulfatase solution (1000 U/mL) was prepared by dissolving 0.366 g in acetate buffer (6 mL). The saccharolactone solution (100 mg/mL) was prepared by dissolving 500 mg in 5mL buffer. The β-glucuronidase inhibitor D-saccharo-1,4-lactone was added to tubes containing the sulphatase preparations because it is known to also contain some β-glucuronidase activity.

The samples were incubated at 37°C for 6 hours. Aliquots from C1 and S2 were taken after 2 hours of incubation and were added to tubes containing para-nitrocatechol sulphate (1 mg/mL) in order to provide a negative and a positive control respectively. The liberation of para- nitrocatechol from para-nitrocatechol sulphate, evidence of an active enzyme preparation was indicated by the production of a yellow colour in S2.

Aliquots of C1, C2 and S3 were taken after 2 hours of incubation and were added to a solution of phenolphtalein glucuronide (10 mg/mL) in order to provide a control for intrinsic glucuronidase activity, a negative control and a positive control respectively. The liberation of phenolphtalein after incubation as indicated by the production of a deep pink colour upon the admixing of a few drops of 1M sodium hydroxide was evidence of an active enzyme preparation.

Enzymatic reactions were stopped by the addition of acetonitrile (400 uL) on ice. Samples were centrifuged at 7000 G for 15 minutes. The supernatant was retained and concentrated under a gentle stream of nitrogen before HPLC analysis. The resulting metabolite profiles were analysed using the HPLC system described previously.

HPLC Analysis of Certified Standards

The characterisation of the fluopicolide metabolites present in urine and faecal extract samples was attempted using the HPLC systems described previously by comparing their retention times with those of available certified standards.

LC/MS - LC/MS/MS analysis

Further characterisation and identification of the metabolites present in urine and faecal extracts was performed using mass spectrometric techniques. The metabolite identifications were performed by using Positive and Negative Ion Electrospray ionisation, Liquid Chromatography-Mass Spectrometry and Liquid Chromatography-Mass Spectrometry-Mass Spectrometry techniques when available, (ESI+/-; LC/MS and LC/MS/MS).

8. Data calculation

The calculation of % dose recovery and compound concentrations, including means and standard deviations, were carried out by use of Debra 5.2a software and Microsoft Excel (version 97 SR-2 for Windows 95) spreadsheet program on a Compaq Prolinea 590 or 5100 personal computer or an IBM ThinkPad portable computer. It is possible that in certain tables the presented means do not calculate exactly from the individual data. This is due to rounding-up differences experienced from the use of the spreadsheet program. The limit of detection was taken as twice the background counting rate obtained from the measurement of blank samples.

II. Results and Discussion

A. Recovery

A summary of the radioactivity in percent of the administered dose is presented below.

Table 5.1.1- 38: Recovery of radioactivity (% dose administered)

Sample	Male		Female	
	Mean	S.D.	Mean	S.D.
Urine	18.81	1.48	21.37	4.64
Faeces	72.37	5.12	68.78	4.70
Cage wash	2.04	1.12	5.27	1.24
Tissue/organ	0.66	0.37	0.46	0.03
Total	93.87	6.78	95.88	1.33

S.D. = Standard deviation

The faeces and urine samples contained almost all of the recovered radioactivity; thus, the metabolism study was performed with these two categories of samples.

B. Metabolism

Urine

Quantification of Urine Metabolites by HPLC

A total of twenty-eight (28) components were observed in the pooled urine samples following administration of the low dose which were numbered in the format "UMET/-" in order of their appearance on the chromatogram i.e. UMET/28 was the least polar component and possessed the longest retention time.

Up to 23 different radioactive fractions were observed in the urine from male rats following a single oral administration of [pyridyl-2,6-I4C]-fluopicolide. Of these only one fraction (UMET/2) accounted for more than 5% of the administered dose with a level of 6.52 %dose. Only three other fractions accounted for between 1 and 4% of the administered radioactivity: UMET/8 (3.59 %dose), UMET/21 (1.0 %dose) and UMET/26 (1.34 %dose). The remaining 19 radioactive fractions accounted for between 0.02 and 0.79 %dose. Hence the male rat was found to be capable of extensively metabolising fluopicolide following oral administration.

The results for the urine from female rats indicated the presence of up to 25 different radioactive fractions following a single oral administration of [pyridyl-2,6-14C]-fluopicolide. Of these only one fraction (UMET/22) accounted for more than 5% of the administered radioactivity representing 6.40 %dose. Seven of the remaining fractions accounted for more than 1% of the administered radioactivity: UMET/2 (1.20 %dose), UMET/13 (1.02 %dose), UMET/18 (2.09 %dose), UMET/21 (1.24 %dose), UMET/23 (1.33 %dose), UMET/24 (1.02 %dose), and UMET/26 (1.69 %dose). The remaining 15 radioactive fractions accounted for between 0.01 and 0.95 %dose. Hence the female rat was also found to be capable of extensively metabolising fluopicolide following oral administration.

Enzymatic hydrolysis of pooled urine samples

Pooled urine samples taken from male and female rats 0-6 h, 6-24 h and 24-48 h were subjected to enzymatic deconjugation. Up to 23 different radioactive fractions were observed in the hydrolysis chromatograms for both males and females. The radioactive fractions observed in the HPLC chromatograms were numbered in the format "MET.U/-" in order of their appearance on the chromatogram i.e. the fraction with the highest number was the least polar component and possessed the longest retention time.

The results from the experiments using urine from the male rats indicated that the major metabolite (MET.U/3) was stable when incubated with either sulphatase or glucuronidase enzyme preparations and is therefore unlikely to be a conjugate.

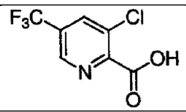
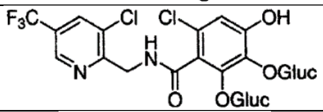
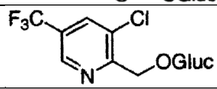
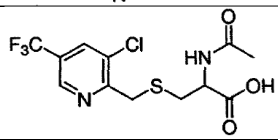
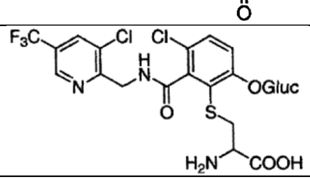
Generally, it appeared that the male urine samples did not contain significant levels of glucuronide conjugates but at least three (MET.U/14, 15 and 16) fractions were sulphate conjugates with the possible addition of MET.U/17. The fractions MET.U/3, 7,20 and 5 were consistently seen to be stable in the presence of the enzyme preparations and are therefore not glucuronide or sulphate conjugates.

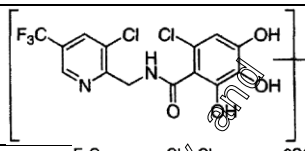
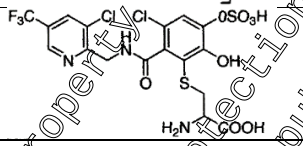
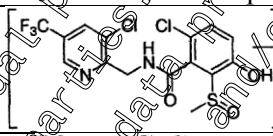
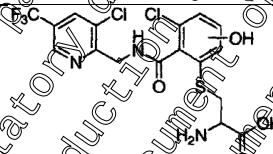
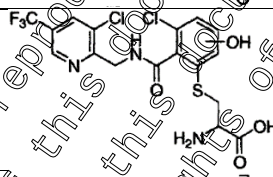
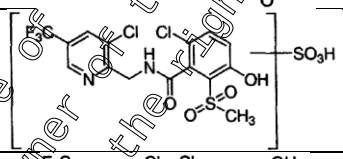
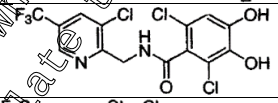
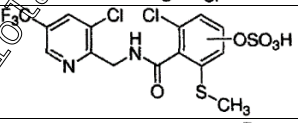
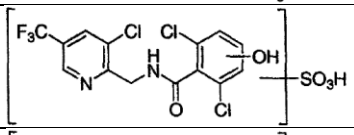
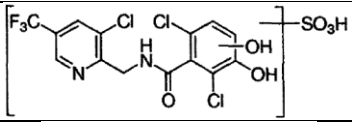
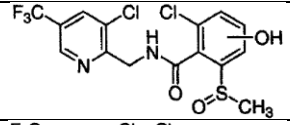
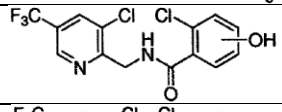
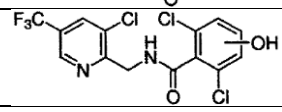
Some differences were observed in the female urine samples compared to the male samples as glucuronide conjugates were observed. The data for the female urine samples is less clear-cut than the male data with several fractions containing both sulphate and glucuronide conjugates (MET.U/15,18,19 and 20). MET.U/17 appears to have been a sulphate conjugate. In terms of stability in the presence of the enzyme preparations the fractions MET.U/2, 6, 7,9, 16 and 22 displayed no decreases upon incubation. MET.U/23 was seen to be an aglycone of a sulphate conjugate (most probably MET.U/17 due to the relative proportions observed).

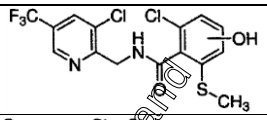
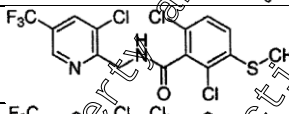
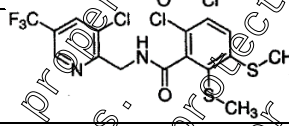
Mass Spectroscopy Results for the Urine samples

The metabolites identified in urine are shown in the table below. In total 92.88% of the radioactivity eliminated in the urine by the male rats has been assigned a proposed structure which accounted for 16.19% of the administered dose. For the females, a total of 82.6% of the radioactivity eliminated in the urine by the female rats has been assigned a proposed structure which accounted for 17.10% of the administered dose.

Table 5.1.1- 39: Metabolites identified in the urine following a single oral low dose of [2,6-pyridyl-^{14}C]-fluopicolide at the nominal rate of 10 mg/kg

UMET/-N°	%Dose		Assigned molecular mass	Proposed structure
	Males	Females		
2	6.52	1.20	225	
4	0.53	0.29	748	
5	0.05	0.21	387	
8	3.59	0.57	356	
9	0.08	0.11	659	

UMET/-Nº	%Dose		Assigned molecular mass	Proposed structure
	Males	Females		
13	0.11	1.02	572	
			563	
			578	Cl_3 but no structure propose
14	0.19	0.08	506	
15	n.d	0.16	483	
17	0.09	0.69	525	
19	0.48	n.d.	522	
21	1.00	1.24	416	
22	0.50	6.40	490	
			478	
23	0.47	1.33	494	
24	0.79	1.02	426	
25	0.22	0.14	364	
26	1.34	1.69	398	

UMET/-Nº	%Dose		Assigned molecular mass	Proposed structure
	Males	Females		
27	0.25	0.95	410	
			428	
28	n.d.	0.01	440	
Total	16.19	17.10		

Faeces

Quantification of Faeces metabolites by HPLC

A total of thirty-one (31) radiolabelled components were observed in the faecal extracts. These components were ascribed numbers in the format "MET.F/1" in order of their appearance on the chromatogram i.e. component MET.F/1 had the shortest retention time.

Up to 31 different radioactive fractions were observed in the faecal extracts from male rats following a single oral administration of [pyridyl-2,6-14C]-fluopicolide. Of these five components (MET.F/8 at 6.74 %dose, MET.F/12 at 5.73 %dose, MET.F/20 at 6.74 %dose, MET.F/21 at 8.36 %dose and MET.F/22 at 5.76 %dose) accounted for more than 5% of the administered dose. MET.F/25 represented 4.32 %dose and MET.F/17, 2.54 %dose. The remaining fractions represented between 0.16 and 2.34% of the administered radioactivity. As seen for the urine metabolites the relatively high number of fractions indicate that the male rat was capable of extensively metabolising [pyridyl-2,6-^{14}C]-fluopicolide following oral administration.

Up to 31 different radioactive fractions were observed in the faecal extracts from female rats following a single oral administration of [pyridyl-2,6-^{14}C]-fluopicolide. Of these five components (MET.F/12 at 5.76 %dose, MET.F/20 at 5.27 %dose, MET.F/21 at 13.65 %dose and MET.F/22 at 9.46 %dose and MET.F/25 at 5.30 %dose) accounted for more than 5% of the administered dose. MET.F/8 represented 3.48%dose and MET.F/17, 3.13 %dose. The remaining fractions represented between 0.06 and 2.36% of the administered radioactivity. As seen for the urine metabolites the relatively high number of fractions indicate that the female rat was capable of extensively metabolising [pyridyl-2,6-^{14}C]-fluopicolide following oral administration.

Comparison of the profile observed in the faecal extracts obtained from the males and females shows that the overall profiles were qualitatively and quantitatively similar. The same major metabolites were present in the samples from both sexes i.e. MET.F/8,12,20,21,22 and 25.

Acidic extracts of faeces

The pooled acidic extracts samples from the 0-24 hour time period contained 2.29% and 3.49% of the dose for the males and females respectively whilst the 24-48 hour samples represented 1.16% (males) and 0.65% (females) of the dose. The 48-72 hour male pooled sample contained 0.21% of the administered radioactivity and was, therefore not investigated byHPLC.

The pooled male faecal acidic extracts were found to contain up to 11 radiocomponents which were assigned numbers in the format "MET.AEF/1" in order of their appearance on the chromatogram i.e. component MET.AEF/1 had the shortest retention time. The major component found in the acidic

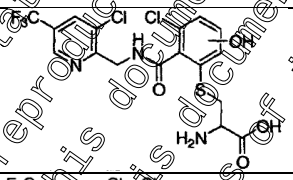
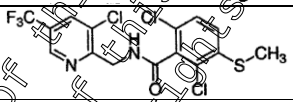
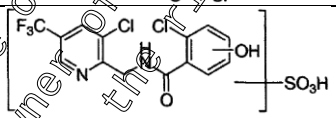
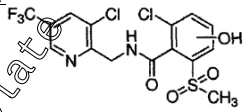
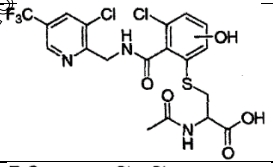
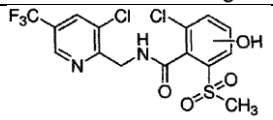
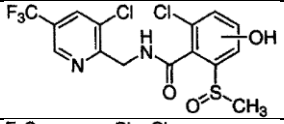
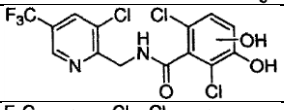
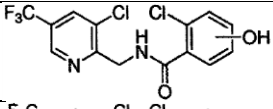
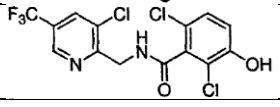
extracts was MET.AEF/12 which represented 1.70 %dose. The remaining components represented between 0.03 to 0.45 %dose.

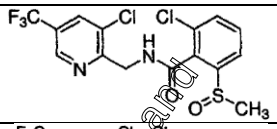
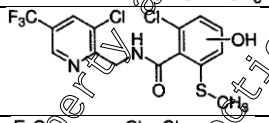
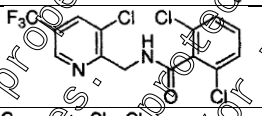
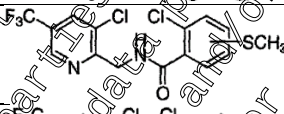
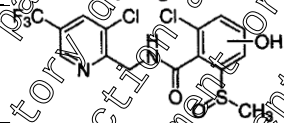
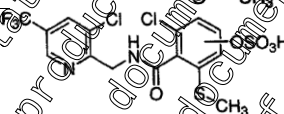
The major component found in the acidic extracts of faeces from female rats was MET.AEF/11 which represented 1.84 %dose. The remaining components represented between 0.02 to 0.61 %dose.

Mass Spectroscopy Results for the Faecal samples

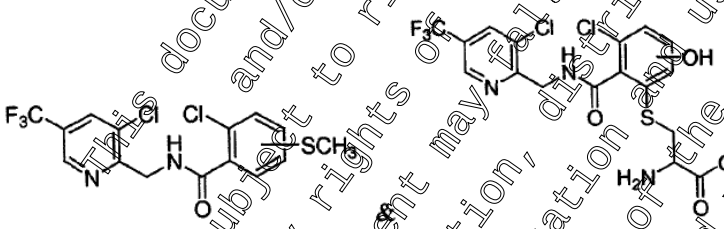
The metabolites identified in faeces are shown in the table below. In total 75.9% of the radioactivity found in the faecal extracts (not including the acidic extracts) of the male rats has been assigned a proposed structure which accounted for 48.2% of the administered dose. For the females a total of 83% of the radioactivity found in the faecal extracts of the female rats has been assigned a proposed structure which accounted for 51.9% of the administered dose.

Table 5.1.1- 40: Metabolites identified in the faeces following single oral low dose of [2,6-pyridyl-^{14}C]-fluopicolide at the nominal rate of 10 mg/kg

MET.F/-N°	%Dose		Assigned molecular mass	Proposed structure
	Males	Females		
4	1.70	0.72	483	
5	1.03	0.97	428	
8	6.74	3.48	444	
9	1.10	0.26	442	
10	1.21	0.65	525	
11	1.26	1.44	442	
12	5.73	5.76	426	
13	1.74	1.63	414	
15	1.21	1.16	364	
16	1.70	2.36	398	

MET.F/-Nº	%Dose		Assigned molecular mass	Proposed structure
	Males	Females		
17	2.54	3.13	410	
20	6.74	5.27	410	
21	8.36	13.65	382	
22	5.76	9.46	394	
22a	0.78	0.82	426	
23	0.62	1.14	492	
Total	48.19	51.90		

The majority of the LC/MS/MS analyses of the acidic extracts of the faeces were unsuccessful. However, the following structures were proposed to be present in the radio component identified as MET.AEF/5 (0.45 %dose in males and 0.54 %dose in females):



Therefore, a total of 51.46% of the administered radioactivity which was eliminated via the faeces by the male rats was assigned a structure. Similarly, a total of 55.88% of the administered radioactivity which was eliminated via the faeces by the female rats was assigned a structure.

By summing the components that were assigned structures in the urine and faeces, a total of 67.64% of the administered radioactivity has been assigned a structure for the males and 72.99% of the administered radioactivity for the females.

C. Metabolic pathway

The proposed metabolic pathway of [Pyridyl-2,6- ^{14}C]-fluopicolide is presented in the following three figures:

Figure 5.1.1-2: Proposed metabolic pathway

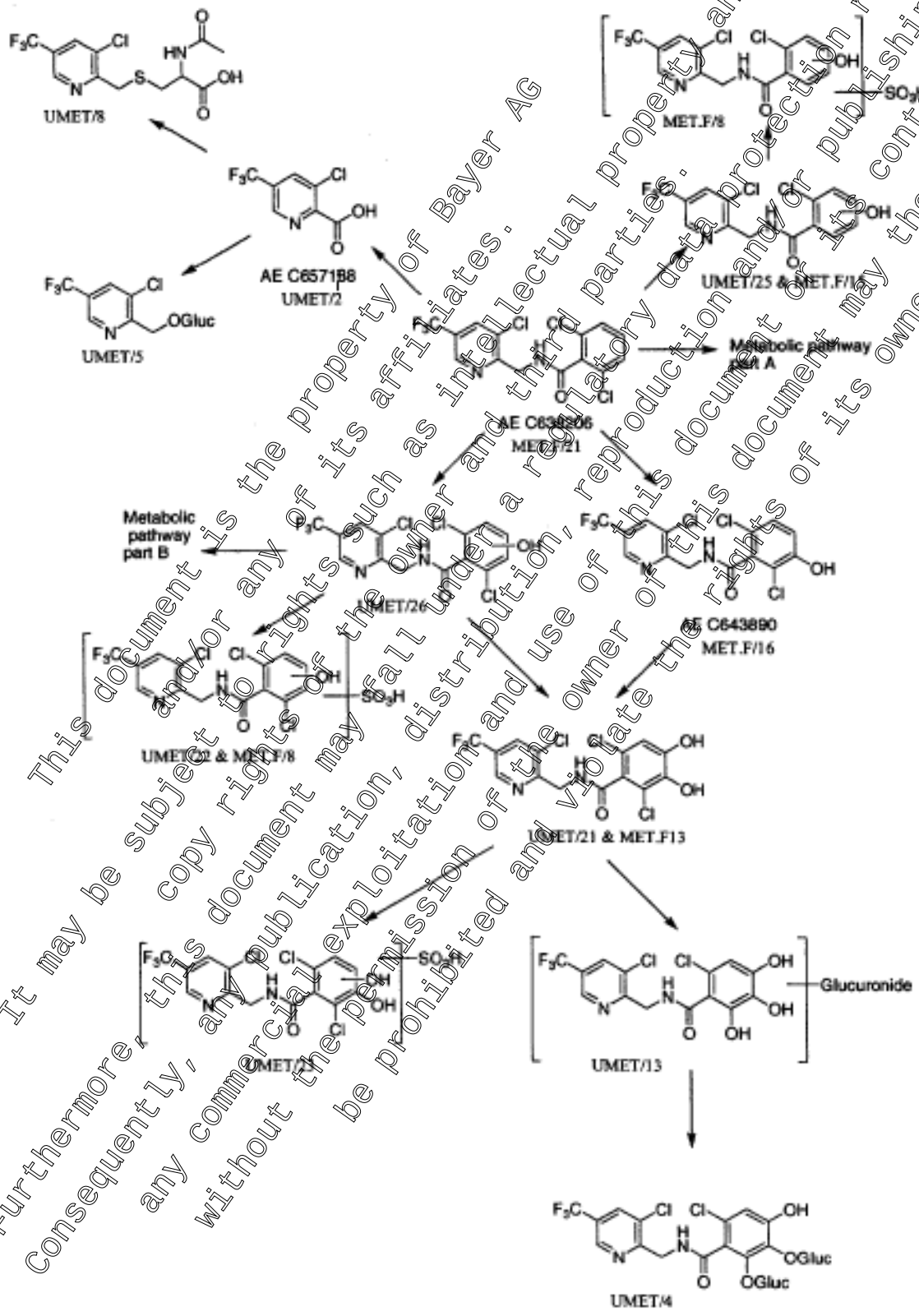


Figure 5.1.1-3: Proposed metabolic pathway – part A

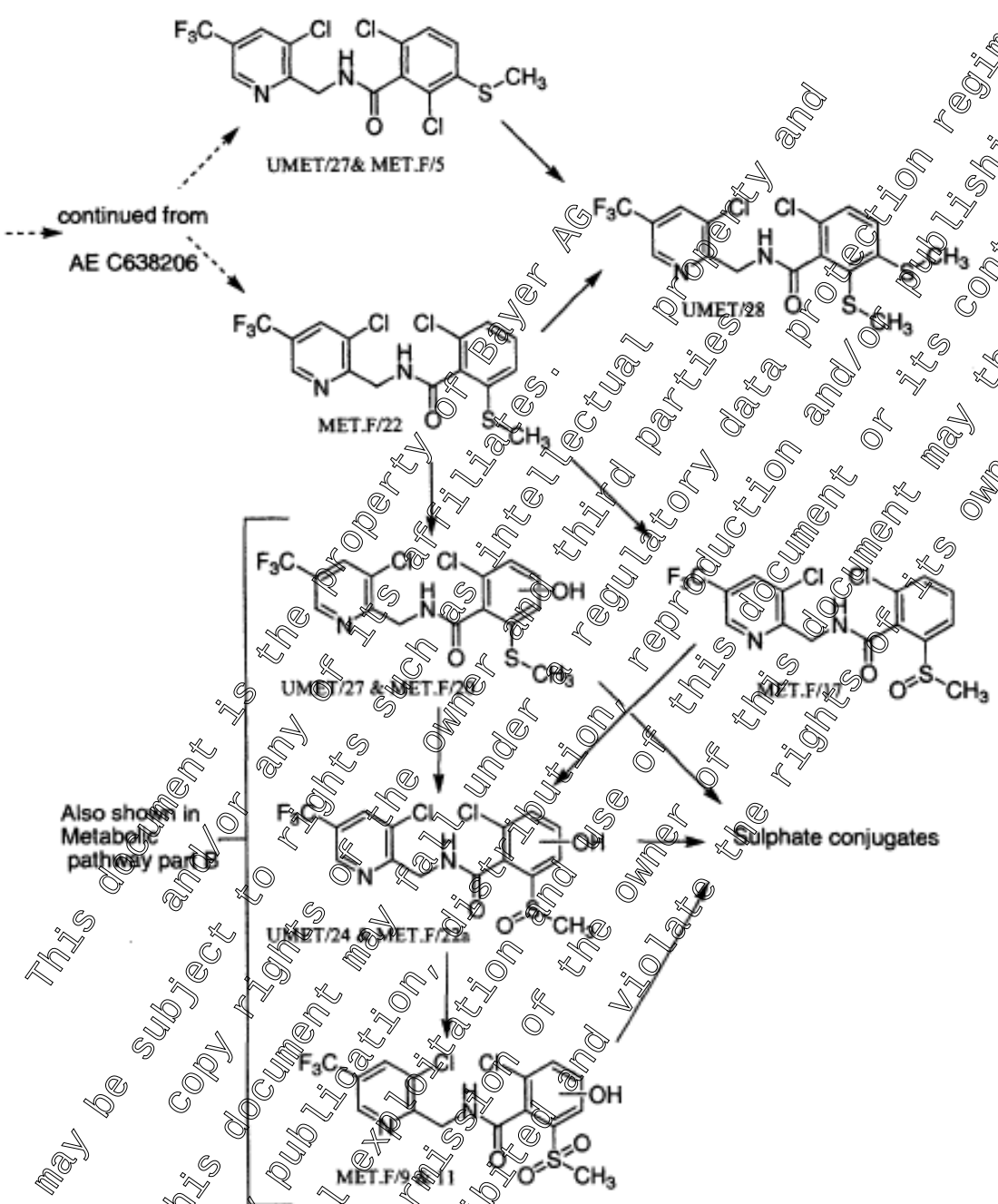
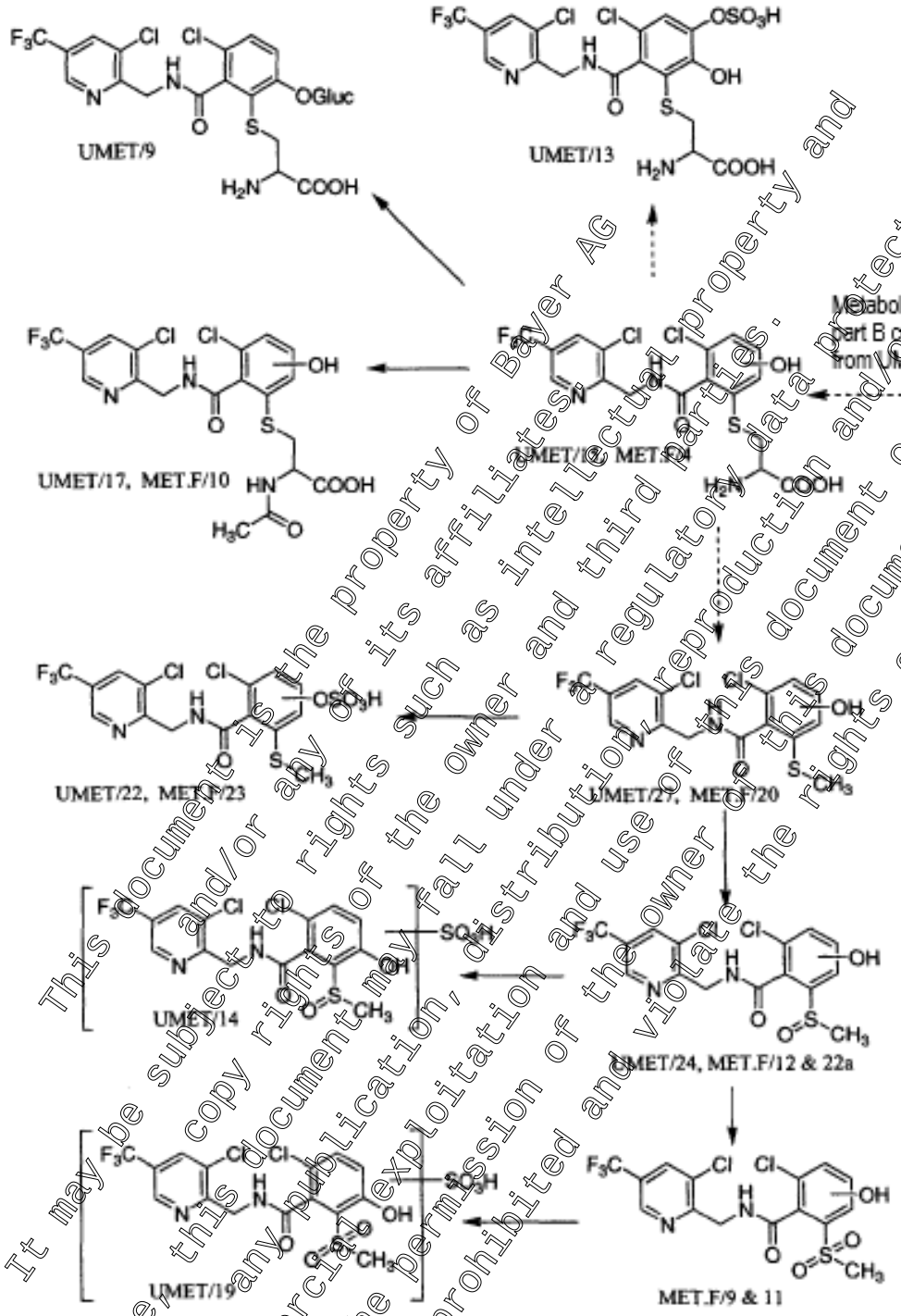


Figure 5.1.1-4: Proposed metabolic pathway – part B



III. Conclusions

Investigations into the metabolism of [Pyridyl-2,6-^{14}C]-fluopicolide in urine and faecal extract samples revealed the presence of up to 28 radioactive components in the urine and up to 31 radioactive components in the faecal extracts. Thus, it appeared that the [Pyridyl-2,6-^{14}C]-fluopicolide was extensively metabolised in the rat following oral dosing.

In total 92.88% of the radioactivity eliminated in the urine by the male rats has been assigned a proposed structure that accounted for 16.19% of the administered dose. For the females a total of 82.6% of the radioactivity eliminated in the urine by the female rats has been assigned a proposed structure which accounted for 17.10% of the administered dose.

A total of 51.46% of the administered radioactivity which had been eliminated via the faeces by the male rats was assigned a structure. Similarly, a total of 55.88% of the administered radioactivity which had been eliminated via the faeces by the female rats was assigned a structure.

By summing the components that were assigned structures in the urine and the faeces, a total of 67.64% of the administered radioactivity has been assigned a structure for the males and 72.99% of the administered radioactivity for the females.

Assessment and conclusion by applicant:
An acceptable study reaching valid conclusions

Data Point:	KCA 5.1.1/10
Report Author:	[redacted]
Report Year:	2004
Report Title:	[Phenyl-U-14C]-AE C638206: Rat metabolism following administration of a single oral high dose
Report No:	C039582
Document No:	M-227025-02-1
Guideline(s) followed in study:	EU (=EEC): 87/302/EEC, Part B; JMAF: 12, Nousan No. 8147, NOV 24, 2000 , 59 Nousan 4200; USEPA (=EPA): OPPTS 870.7485
Deviations from current test guideline:	not specified
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

The metabolism of fluopicolide in the rat was investigated with radiolabelled fluopicolide ([phenyl U-^{14}C]-fluopicolide).

A single oral gavage dose of [phenyl U-^{14}C]-fluopicolide was administered to 4 male and 4 female Sprague Dawley rats at a nominal dose of 100 mg/kg bw (Single Oral High Dose). Samples of urine were collected at 0 – 6 h, 6 – 24 h and then at 24-hourly intervals up to 168 h and samples of faeces were collected at 24-hourly intervals up to 168 h. These urine and faeces samples were obtained from the rat ADE study Totis, M. (2001), M-204781-01-1 reported at KCA 5.1.1/02.

The radioactivity in pooled samples of urine (0 – 6 h, 6 – 24 h and 24 – 48 h) and faeces (0 – 24 h and 24 – 48 h) from the male and female rats was characterised by radio-HPLC and by LC-MS and LC-MS/MS. In total, up to 46 radioactive components were detected in the urine samples and up to 14 radioactive components were detected in the faecal extracts, thus indicating that [phenyl U-^{14}C]-fluopicolide was extensively metabolised in the rat following oral dosing.

In the urine samples, up to 33 different radioactive fractions were observed in the urine of the male rats and 42 different radioactive fractions were observed in the urine of the female rats. Qualitatively, the metabolite profiles were similar between the sexes although a greater number of metabolites were observed in the female urine compared to the male urine and some quantitative differences were also observed. In the male rats, the most significant component was UMET/3 which accounted for 0.962% of the dose; six metabolites each individually amounted to between 0.207 – 0.392% of the dose (UMET/1, UMET/2, UMET/4, UMET/6, UMET/30 and UMET/36); a further seven metabolites were found to account for between 0.100 – 0.179% of the dose (UMET/5, UMET/12, UMET/14, UMET/18, UMET/27, UMET/32 and UMET/39) and the remaining 19 detectable fractions each accounted for 0.007 – 0.099% of the dose. In the female rats, the most significant component was UMET/36 which accounted for 1.534% of the dose; four metabolites each individually amounted to between 0.289 – 0.327% of the dose (UMET/1, UMET/24, UMET/26 and UMET/32); a further seven metabolites were found to account for between 0.102 – 0.215% of the dose (UMET/3, UMET/4, UMET/25, UMET/39, UMET/41, UMET/44 and 45) and the remaining 28 detectable fractions each accounted for 0.001 – 0.089% of the dose. Structures were able to be proposed for a number of the detected fractions. UMET/1 was assigned a mass of 188 m/z; UMET/4 was proposed as the mercapturic acid conjugate of a mono-hydroxylated AE C653711 (M01, also known as BAM); UMET/30 comprised of two components which were assigned as sulfone and sulfoxide derivatives (and their sulphate conjugates) following substitution of a chlorine atom on the phenyl ring by glutathione and subsequent degradation to the cysteine conjugate; UMET/36 was proposed as the equivalent sulphide; UMET/24 comprised of two components, one component and UMET/26 were proposed to be a glucuronide conjugate of parent material that had undergone hydrolytic removal of one of the chlorine atoms from the phenyl ring and hydroxylations on the 3 and 4 positions on the ring whilst the second UMET/24 component was assigned to the sulphate and cysteine conjugate.

In the faecal samples, up to 11 different fractions were observed in the faeces of the male and female rats and the metabolic profiles were observed to be similar between the sexes. The most significant component was FMET/11, accounting for 79.97% and 81.56% of the administered dose in the male and female rats, respectively. FMET/10 amounted to 2.16% of the dose in male rats and 2.33% of the dose in female rats; FMET/9 amounted to 1.55% of the dose in male rats and 1.22% of the dose in female rats; the remaining fractions represented 0.07 – 0.62% of the administered radioactivity in male rats and 0.15 – 0.76% in female rats. FMET/11 was identified as being parent fluopicolide; FMET/10 was identified as the S-methyl derivative following the loss of a chlorine atom from the phenyl ring (suggestive of a similar route of formation as UMET/30 and UMET/36); FMET/9 was assigned to the hydroxylated FMET/10.

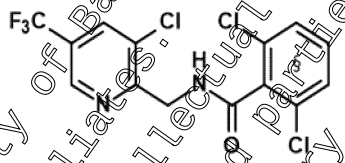
40.76% of the radioactivity eliminated in urine by the male rats and 76.95% by the female rats was able to be assigned to proposed structures which accounted for 1.80% and 4.41% of the administered dose, respectively. 99.54% of the radioactivity eliminated in faeces by the male rats and 98.96% by the female rats was able to be assigned to proposed structures which accounted for 85.40% and 86.38% of the administered dose, respectively. Overall, a total of 87.2% of the administered radioactivity was assigned a structure for the males and 90.8% of the administered radioactivity for the females.

Most of the proposed metabolites contained both aromatic rings suggesting that there is not significant cleavage between the rings. Most metabolites were derived from the parent molecule by hydroxylation then conjugation in the phenyl ring and/or by substitution of a chlorine atom in the phenyl ring by glutathione followed by subsequent degradation to the cysteine conjugate. No significant metabolism in the pyridyl ring was observed. A metabolic pathway for fluopicolide in the rat is proposed.

I. Materials and methods

A. Materials

1. Test Material:

IUPAC Name	2,6-dichloro-N-[[3-chloro-5-(trifluoromethyl)-2-pyridinyl]methyl]-benzamide
CAS Name	benzamide, 2,6-dichloro-N-[[3-chloro-5-(trifluoromethyl)-2-pyridinyl]methyl]
Code name	AE C638206
Common name	Fluopicolide
Empirical formula	$C_{14}H_8Cl_3F_3N_2O$
CAS Number	239110-15-7
Molar mass	383.59 g/mol
Chemical structure	 * denotes position of label
Radiolabelled test material	[phenyl-U-^{14}C]-fluopicolide*
Lot number	901-CU-2
Radiochemical purity	> 99% (by HPLC)
Specific radioactivity	59.5 mCi/mmole or 2.202 GBq/mmole
Specific radioactivity of dose suspensions	Checked by HPLC and radio assay
Stability of test compound	Stable at -20°C
Non-radiolabelled test material	Fluopicolide
Lot number	R001737
Purity	99.3%

*referred to as [phenyl-U-14C]-AE C638206 in the report

2. Vehicle: Aqueous methyl cellulose (0.75% w/w)

3. Test animals:

Species: Rat
Strain: Sprague Dawley, CD
Age: Not stated
Weight at dosing: 151 – 175g
Source: [redacted]
Acclimation period: 7 days
Identification: Ear tattoo
Diet: Certified rodent diet M20 (Pietrement, Provins, France)
Water: Provided ad libitum (filtered, softened water from municipal supply)
Housing: Housed in metal wire-mesh bottomed cages in metal trays containing absorbent paper during acclimatisation and in specialised metabolism cages thereafter

Environmental conditions:

Temperature: 22 ± 2°C
Humidity: 55 ± 15%
Photoperiod: 12 hours
Air changes: Average 15 changes/hour

4. Preparation of dosing solutions

The doses were prepared by mixing weighed amounts of radiolabelled and non-radiolabelled material in acetonitrile to achieve a homogenous solution at the desired specific radioactivity. The solution was then gently evaporated under a stream of nitrogen, appropriate weights of aqueous methyl cellulose (0.75% w/w) added and the resulting solution ground to produce a suspension. The dose suspension was of sufficient quantity and concentration to provide a dose of 1g per 200 g rat body weight. The dose suspensions for the low and high dose groups were prepared on the day of dosing and assayed by HPLC for active ingredient content and for radioactivity content (by LSC) before, during and following the dosing procedure.

B. Study design and methods

1. In life dates: 12th September 2000 to 19th April 2001 (in-life phase), 28th May 2001 – 11th April 2002 (analytical phase).

2. Dose regimen and design of tests

Administered single dose of [phenyl-U-^{14}C]-fluopicolide, route (experiment)	Number of rats and sex	Collection of samples during the test and at sacrifice	Duration
100 mg/kg bw, oral (single high dose)	4 males 4 females	Urine, faeces, cage wash, blood plasma, organs, tissues, carcass	7 days

The rats each received a single dose of [phenyl-U-^{14}C]-fluopicolide at a target dose of 100 mg/kg bw/d. Urine and faeces samples were collected at intervals until sacrifice at 168-hours, when the blood, organs, tissues and carcass were collected.

3. Dosing

Adequate volumes of the suspension of the test compound in aqueous methyl cellulose (0.75% w/w) were administered to fasted (18-hours) rats via a single oral gavage dose. The doses for each individual animal were calculated based on the weights of the animal prior to dosing (food was replaced one-hour post dose and remained available ad libitum throughout the remainder of the study).

4. Collection of excreta

Following administration of the radiolabelled test substance, animals were kept in specialised individual metabolism units (Jencon's metabowls Mk III), which permitted the separate quantitative collection of urine and faeces, for a maximum of 168 hours following dosing. Urine was collected at 0-6 and 6-24 hours and subsequently at 24-hour intervals following dosing, whilst faeces was collected at 24-hour intervals following dosing. Cages were washed with distilled water for each 24-hour period post-dose and then with acetonitrile at the end of the in-life phase; cage washes were retained for analysis. Both urine and faeces were collected frozen over solid CO_2 in weighed polystyrene boxes; carded cotton protected the samples from light. Note, these samples were obtained from the rat ADE study [redacted] 2001; M-204781-01-1 reported at KCA 5.1.1/02.

5. Sacrifice

All animals were killed by exsanguination under Imogene 500 anaesthesia, at 7 days following dose administration.

6. Radioassay

Pooled samples of urine, cage washes and aqueous homogenates of faeces (50% w/v) from the male and female groups were radioassayed by liquid scintillation counting (LSC) in Ultima Gold liquid scintillation cocktail (10mL) after stabilising to ambient temperature and reduced light intensity.

7. Preparation of pooled samples

Aliquots of the 0 – 6 h, 6 – 24 h and 24 – 48 h pooled urine samples from the male and female groups were concentrated under a stream of nitrogen gas, a small volume of methanol was added and the samples were then centrifuged. The resulting supernatants were radioassayed by LSC to determine that they were sufficiently concentrated for adequate chromatography (50,000 – 80,000 dpm in less than 200 µL).

Aliquots of the 0 – 24 h, 24 – 48 h pooled faeces samples from the male and female groups were extracted with acetonitrile using an Ultra Turrax homogeniser and then centrifuged. The resulting pellet was further extracted with acetonitrile, acetonitrile: water (70:30 v/v), chloroform: acetonitrile: water (80:20 v/v, pH 11) and then finally acetonitrile:water (60:40 v/v, pH 4) using an ultrasonic bath and centrifugation at each step. The combined supernatants were concentrated under a stream of nitrogen gas and radioassayed by LSC.

8. Characterisation

The quantitative profiles of the urine samples were obtained using radio-HPLC. This method comprised of a Phenomenex LUNA C18 (5 µm) 250 × 4.6 mm column eluted with a gradient of 95% A:5% B to 55% A:45% B over 80 min where mobile phase A comprised 10 mM ammonium acetate/0.1% tetrahydrofuran (pH 5.5) and mobile phase B comprised acetonitrile/0.5% tetrahydrofuran. Detection was by UV absorption at 254 nm and with a flow-through radioactivity detector with a 200 µL CaFl cell.

The quantitative profiles of the faeces extracts were obtained using radio-HPLC. This method comprised of a Phenomenex LUNA C18 (5 µm) 250 × 4.6 mm column eluted with a gradient of 95% A:5% B to 55% A:45% B over 80 min where mobile phase A comprised 10 mM ammonium acetate/0.1% tetrahydrofuran (pH 5.5) and mobile phase B comprised acetonitrile/0.5% tetrahydrofuran. Detection was by UV absorption at 254 nm and with a flow-through radioactivity detector with a 400 µL lithium glass flow cell.

Identification of parent fluopicolide and the metabolites M-06 (AE C643890), M-02 (AE C657188), M-01 (AE C653711) and 2-chlorobenzamide was attempted by retention time matching to certified reference standards using the radio-HPLC system.

Further characterisation and identification were performed by LC-MS and LC-MS/MS. This method comprised of a Phenomenex LUNA C18 (5 µm) 250 × 4.6 mm column eluted with a gradient of 95% A:5% B to 55% A:45% B over 80 min where mobile phase A comprised 10 mM ammonium acetate/0.1% tetrahydrofuran (pH 5.5) and mobile phase B comprised acetonitrile/0.5% tetrahydrofuran. Detection was by UV absorption using a DAD over the range 190 – 600 nm and by ESI mass spectrometry in the positive and negative ion modes. Structures were proposed on the basis of identified molecular ions and fragmentation patterns.

II. Results and Discussion

A. Recovery

In males and females, the majority of the elimination of [phenyl-U-^{14}C]-fluopicolide was via the faeces, with a corresponding lower amount being excreted via the urine (see table below).

Table 5.1.1- 41: Excretion of radioactivity at time intervals, expressed as % dose administered

Dose, route	100 mg/kg bw		100 mg/kg bw	
Experiment	single high dose		single high dose	
Duration, sex	168 h, 4 males		168 h, 4 females	
Time period (h)	Mean	SD	Mean	SD
Radioactivity in faeces (% of dose administered, cumulative)				
0-24	79.79	6.25	78.39	8.48
0-48	85.79	6.07	87.29	7.94
0-72	86.82	6.20	87.99	7.89
0-96	87.22	6.27	88.20	8.07
0-120	87.33	6.30	88.24	8.07
0-144	87.43	6.32	88.26	8.06
0-168	87.46	6.32	88.28	8.06
Radioactivity in urine (% of dose administered, cumulative)				
0-6	0.55	0.21	0.54	0.16
0-24	2.94	1.24	3.62	1.09
0-48	4.42	1.73	5.73	0.96
0-72	4.95	2.02	6.24	0.96
0-76	5.19	2.22	6.39	0.99
0-120	5.29	2.30	6.48	0.99
0-144	5.35	2.34	6.54	1.00
0-168	5.39	2.35	6.58	1.00

In total, in the male rats, 92.85% of the administered dose was excreted over the 168 h period following dosing whilst in female rats, 94.86% of the administered dose was excreted. The excretion was fast and essentially complete 24 – 48 h post-administration. As such, only samples of urine over the intervals 0 – 6 h, 6 – 24 h and 24 – 48 h, and samples of faeces over the intervals 0 – 24 h and 24 – 48 h were selected for characterisation and identification.

The mean recovery of the sample work-up of the urine samples was 111 – 130% whilst the faeces extraction procedure recovered 82.5 – 97.3% of the radioactivity for characterisation.

B. Metabolite quantification

1. Urine

Up to 33 different radioactive fractions were observed in the urine of the male rats and 42 different radioactive fractions were observed in the urine of the female rats. Qualitatively, the metabolite profiles were similar between the sexes although a greater number of metabolites were observed in the female urine compared to the male urine and some quantitative differences were also observed.

Peaks in the profiles of the urine samples were sequentially named UMET/#, based upon their respective elution times. In the male rats, the most significant component was UMET/3 which accounted for 0.962% of the dose; six metabolites each individually amounted to between 0.207 – 0.392% of the dose (UMET/1, UMET/2, UMET/4, UMET/6, UMET/30 and UMET/36), a further seven metabolites were found to account for between 0.100 – 0.179% of the dose (UMET/5, UMET/12, UMET/14, UMET/18, UMET/27, UMET/32 and UMET/39), and the remaining 19 detectable fractions each accounted for 0.007 – 0.099% of the dose. In the female rats, the most significant component was UMET/36 which accounted for 1.534% of the dose; four metabolites each individually amounted to between 0.289 – 0.327% of the dose (UMET/1, UMET/24, UMET/26 and UMET/32); a further seven metabolites were found to account for between 0.102 – 0.215% of the dose (UMET/3, UMET/4, UMET/25, UMET/39, UMET/41, UMET/44 and 45), and the remaining 28 detectable fractions each accounted for 0.001 – 0.089% of the dose. The table below presents the quantification data for the metabolites in the urine from the male and female rats.

Table 5.1.1- 42: Quantification of metabolites in urine from male and female rats following a single oral dose of [phenyl-U-^{14}C]-fluopicolide at the rate of 100 mg/kg b/w (results are expressed in terms of percentage of administered radioactivity)

Metabolite ID	Males				Females			
	0-6 h	6-24 h	24-48 h	Sum	0-6 h	6-24 h	24-48 h	Sum
UMET/1	0.13	0.19	0.067	0.39	0.057	0.182	0.088	0.33
UMET/2	n.d.	0.24	n.d.	0.24	0.006	0.029	0.051	0.09
UMET/3	0.02	0.71	0.235	0.96	n.d.	0.084	0.067	0.15
UMET/4	n.d.	n.d.	0.207	0.21	0.016	0.153	0.045	0.22
UMET/5	0.03	0.08	n.a.	0.11	0.014	n.d.	0.043	0.06
UMET/6	0.09	0.06	0.195	0.35	0.03	0.023	n.d.	0.05
UMET/7	0.03	n.d.	n.d.	0.03	0.019	n.d.	n.d.	0.02
UMET/8	n.d.	n.d.	n.d.	n.a.	0.002	n.d.	n.d.	0
UMET/9	n.d.	0.02	n.d.	0.02	n.d.	n.d.	n.d.	n.a.
UMET/10	n.d.	0.03	n.d.	0.03	n.d.	0.022	n.d.	0.02
UMET/11	0.02	0.04	n.a.	0.06	n.d.	n.d.	n.d.	n.a.
UMET/12	0.03	0.07	0.067	0.16	0.022	0.033	n.d.	0.06
UMET/13	n.d.	n.d.	n.d.	n.a.	n.d.	0.014	n.d.	0.01
UMET/14	0.02	0.13	0.027	0.18	n.d.	0.047	n.d.	0.05
UMET/15	n.d.	0.02	n.d.	0.02	n.d.	0.021	n.d.	0.02
UMET/16	0.02	0.02	n.d.	0.04	0.011	0.02	n.d.	0.03
UMET/17	n.d.	0.01	n.d.	0.01	n.d.	n.d.	n.d.	n.a.
UMET/18	0.02	0.07	0.015	0.11	0.01	0.055	n.d.	0.07
UMET/19	n.d.	0.03	0.011	0.04	n.d.	0.032	n.d.	0.03
UMET/20	n.d.	n.d.	n.d.	n.a.	0.012	0.047	n.d.	0.06
UMET/21	0.01	0.04	0.014	0.06	0.012	0.045	n.d.	0.06
UMET/22	n.d.	n.d.	n.d.	n.a.	n.d.	0.016	n.d.	0.02
UMET/23	n.d.	n.d.	n.d.	n.a.	n.d.	0.02	n.d.	0.02
UMET/24	0.01	0.06	0.021	0.09	0.005	0.298	0.012	0.32
UMET/25	n.d.	0.03	n.d.	0.03	0.016	n.d.	0.102	0.12
UMET/26	0.01	0.04	n.d.	0.05	0.018	0.26	0.029	0.31
UMET/27	n.d.	0.1	n.d.	0.1	0.057	0.026	0.006	0.09
UMET/28	0.02	0.03	n.d.	0.05	0.027	0.391	0.082	0.5
UMET/29	n.d.	n.d.	n.d.	n.a.	0.044	n.d.	n.d.	0.04
UMET/30	n.d.	0.12	0.183	0.3	0.018	0.184	0.267	0.47
UMET/31	n.d.	n.d.	n.d.	n.a.	0.011	0.025	n.d.	0.04
UMET/32	0.06	0.08	0.016	0.15	0.077	0.177	0.035	0.29
UMET/33	n.d.	n.d.	n.d.	n.a.	n.d.	0.015	0.012	0.03
UMET/34	n.d.	n.d.	0.074	0.07	0.008	0.019	0.036	0.06
UMET/35	n.d.	n.d.	n.d.	n.a.	0	0	0.001	0
UMET/36	n.d.	0.1	0.131	0.23	0.008	n.d.	0.88	1.53
UMET/37	n.d.	n.d.	n.d.	n.a.	0.015	n.d.	n.d.	0.02
UMET/38	n.d.	n.d.	n.d.	n.a.	n.d.	0.007	n.d.	0.01
UMET/39	0.01	0.06	0.082	0.15	0.013	0.052	0.082	0.15
UMET/40	n.d.	n.d.	0.021	0.02	n.d.	n.a.	0.011	0.01
UMET/41	n.d.	n.d.	0.007	0.01	0.012	0.019	0.085	0.12
UMET/42	n.d.	n.d.	0.035	0.04	n.d.	n.d.	n.d.	n.a.
UMET/43	n.d.	n.d.	n.d.	n.a.	n.d.	n.d.	0.016	0.02
UMET/44	0.02	0.03	0.05	0.1	n.d.	0.073	0.082	0.16
UMET/45	n.d.	n.d.	0.02	0.02	n.d.	0.026	0.076	0.1
UMET/46	n.d.	n.d.	n.d.	n.a.	n.d.	0.019	n.d.	0.02
Total	0.55	2.39	1.484	4.42	0.543	2.432	2.108	5.73

n.d. = not detected, n.a. = not applicable.

2. Faeces

Up to 11 different fractions were observed in the faeces of the male and female rats and the metabolic profiles were observed to be similar between the sexes. Peaks in the profiles of the faeces samples were sequentially named FMET/#, based upon their respective elution times. The most significant component was FMET/11, accounting for 79.97% and 81.56% of the administered dose in the male and female rats, respectively. FMET/10 amounted to 2.16% of the dose in male rats and 2.33% of the dose in female rats; FMET/9 amounted to 1.55% of the dose in male rats and 1.22% of the dose in female rats; the remaining fractions represented 0.07 – 0.62% of the administered radioactivity in male rats and 0.15 – 0.76% in female rats. The table below presents the quantification data for the metabolites in the faeces from the male and female rats.

Table 5.1.1- 43: Quantification of metabolites in faeces from male and female rats following a single oral dose of [phenyl-U-^{14}C]-fluopicolide at the rate of 100 mg/kg b/w (results are expressed in terms of percentage of administered radioactivity)

Metabolite ID	Males			Females		
	0-24 h	24-48 h	Sum	0-24 h	24-48 h	Sum
FMET/1	n.d.	0.201	0.200	n.d.	0.184	0.180
FMET/2	n.d.	0.113	0.110	n.d.	0.152	0.150
FMET/3	n.d.	0.195	0.190	n.d.	n.d.	n.a.
FMET/4	n.d.	n.d.	n.a.	n.d.	0.218	0.220
FMET/5	n.d.	0.620	0.620	n.d.	n.d.	n.a.
FMET/6	n.d.	0.431	0.430	n.d.	0.761	0.760
FMET/7	n.d.	n.d.	n.a.	n.d.	0.186	0.190
FMET/8	n.d.	0.275	0.280	n.d.	0.332	0.330
FMET/9	n.d.	1.547	1.550	n.d.	1.219	1.220
FMET/10	1.789	0.380	2.160	1.870	0.457	2.330
FMET/11	78.010	1.958	79.970	76.720	4.839	81.560
FMET/12	n.d.	0.206	0.210	n.d.	0.167	0.170
FMET/13	n.d.	n.d.	n.a.	n.d.	0.184	0.180
FMET/14	n.d.	0.071	0.070	n.d.	n.d.	n.a.
Total	79.791	5.996	86.790	78.591	8.699	87.290

n.d. = not detected, n.a. = not applicable

C. Metabolite identification

1. Urine

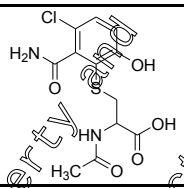
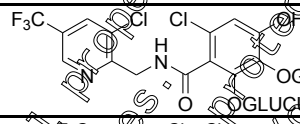
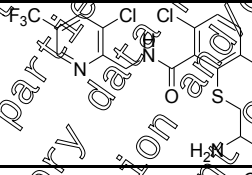
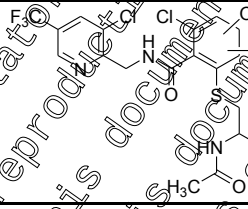
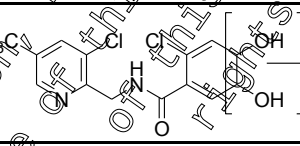
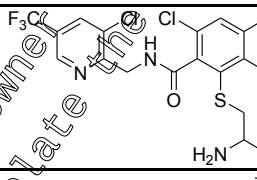
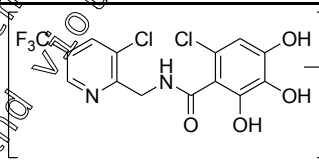
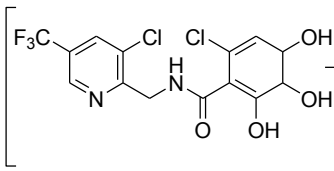
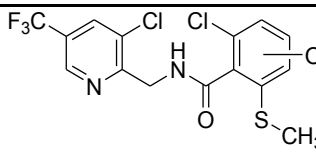
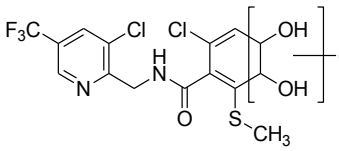
By retention time matching, no parent fluopicolide was observed in the urine samples. UMET/9 had a similar retention time to 2-chlorobenzamide, but this identification was not supported by subsequent LC-MS analysis and so this metabolite is characterised only. UMET/12 had a similar retention time to M-01 (AE C653711) but this identification was not supported by subsequent LC-MS analysis and so this metabolite is characterised only. UMET/44 had a similar retention time to M-06 (AE C643890) and this identification was supported by LC-MS.

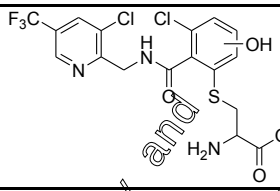
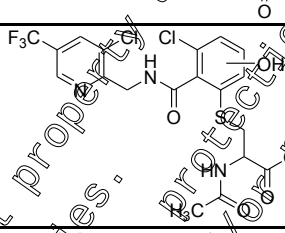
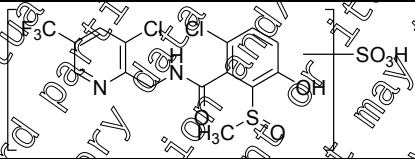
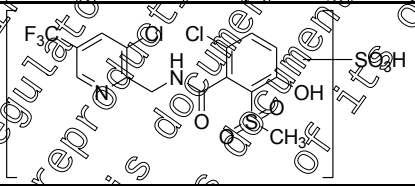
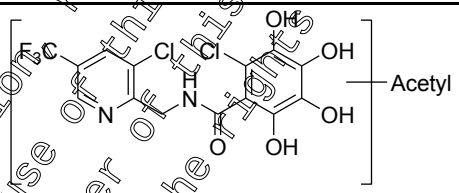
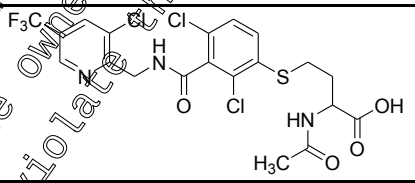
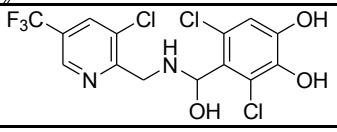
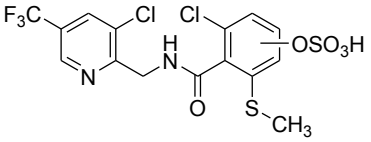
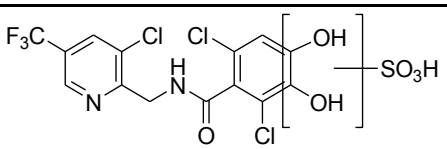
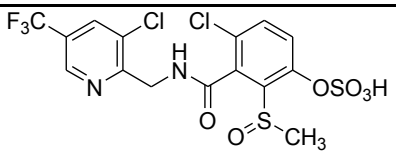
Analysis by LC-MS/MS enabled structures to be proposed for a number of the fractions, this analysis also indicated than some peaks in the profile contained more than one component but since 46 different fractions had already been resolved, limited improvement to the profile resolution by altering the chromatography would be expected. The assignments made for the more significant components in the urine profile are summarised below:

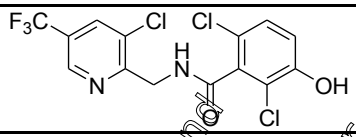
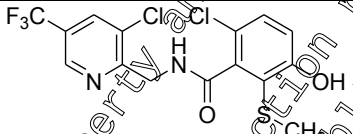
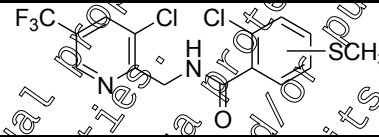
- UMET/1 was assigned a mass of 188 m/z but no structure could be proposed
- UMET/4 was proposed as the mercapturic acid conjugate of a mono-hydroxylated M-01 (AE C653711, also known as BAM).
- UMET/30 comprised of two components which appeared to have resulted from initial substitution of a chlorine atom on the phenyl ring by glutathione (catalysed by glutathione-S-transferase) followed by degradation to the cysteine conjugate. These conjugates could then be cleaved *via* β-elimination by cysteine conjugate β-lyases leaving a resulting thiol that could serve as the methyl acceptor. A mechanism known as the thiomethyl shunt. The resulting S-methyl could then have been oxidised to provide the proposed sulfone and sulfoxide structures. Both components also possessed sulphate conjugates.
- UMET/36 was related to the components found in UMET/30 as it was the equivalent sulphide.
- UMET/24 was found to contain two components, one was proposed to be the glucuronide conjugate of fluopicolide that undergone hydrolytic removal of one of the chlorine atoms from the phenyl ring and hydroxylations on the 3 and 4 positions on the ring, the other component was proposed to be a cysteine and sulphate conjugate of fluopicolide following the same hydrolytic removal of one of the chlorine atoms from the phenyl ring and hydroxylations on the 3 and 4 positions on the ring.
- UMET/26 was proposed to have a similar structure to the glucuronide conjugate proposed for one of the UMET/24 components.

The overall identification of the metabolites in the urine from the male and female rats is summarised in the table below.

Table 5.1.1- 44: Identification of metabolites in urine from male and female rats following a single oral dose of [Phenyl-U-^{14}C]-fluopicolide at a rate of 100 mg/kg b/w.

Metabolite ID	%dose		Assigned molecular mass	Proposed structure
	Males	Females		
UMET/4	0.207	0.215	332	
UMET/14	0.179	0.047	748	
UMET/18	0.108	0.066	659	
UMET/20	n.d.	0.059	543	
UMET/21	0.061	0.057	558	
UMET/24	0.090	0.316	563	
			572	
UMET/26	0.050	0.307	574	
UMET/27	0.100	0.089	586	
			588	

Metabolite ID	%dose		Assigned molecular mass	Proposed structure
	Males	Females		
UMET/28	0.051	0.501	483	
			525	
UMET/30	0.298	0.470	506	
			522	
UMET/31	n.d.	0.036	454	
			543	
UMET/32	0.153	0.289	416	
UMET/36	0.232	1.534	490	
UMET/39	0.154	0.147	494	
			506	

Metabolite ID	%dose		Assigned molecular mass	Proposed structure
	Males	Females		
UMET/44	0.099	0.155	398	
UMET/45	0.020	0.102	410	
UMET/46	n.d.	0.019	394	
Total	1.802	4.409		

n.d. = not detected

The investigations by LC/MS and LC-MS/MS permitted a total of 40.76% of the radioactivity eliminated in the urine over the 0 – 48 h period post-dose by the male rats to be assigned a proposed structure which accounted for 1.80% of the administered dose. For the females, a total of 76.95% of the radioactivity eliminated in the urine over the 0 – 48 h period post-dose was assigned to a proposed structure which accounted for 4.41% of the administered dose.

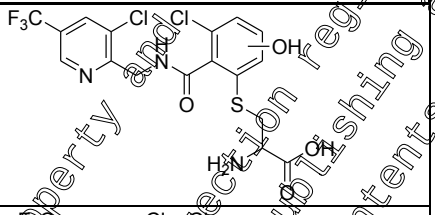
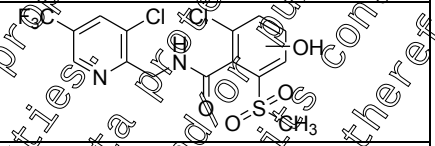
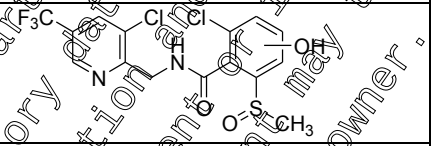
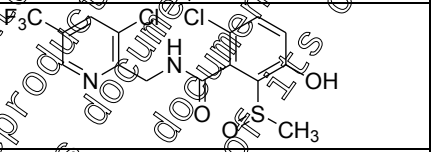
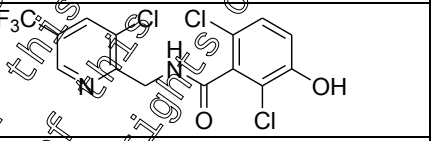
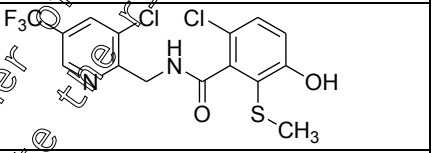
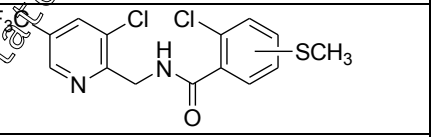
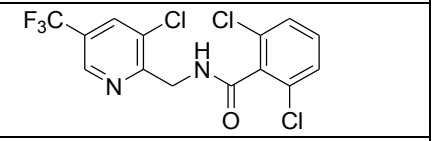
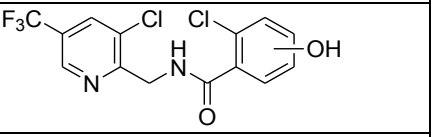
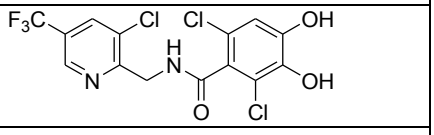
2. Faeces

By retention time matching, parent fluopicolide was assigned to FMET/11 and this assignment was confirmed by mass spectroscopy. FMET/8 had a similar retention time to M-06 (AE C643890), and this assignment was confirmed by mass spectroscopy.

Analysis by LC-MS and LC-MS/MS enabled structures to be proposed for a number of the fractions. FMET/10 was identified as being parent material which has lost a chlorine atom on the phenyl ring with the addition of an S-methyl group, most likely forming by the same route as that proposed for UMET/30 and UMET/36. FMET/9 was identified as a hydroxylated form of FMET/10.

The overall identification of the metabolites in the faeces from the male and female rats is summarised in the table below.

Table 5.1.1- 45: Identification of metabolites in faeces from male and female rats following a single oral dose of [phenyl-U-^{14}C]-fluopicolide at a rate of 100 mg/kg b/w.

Metabolite ID	%dose		Assigned molar mass	Proposed structure
	Males	Females		
FMET/1	0.20	0.18	483	
FMET/3	0.19	n.d.	442	
FMET/5	0.62	n.d.	426	
FMET/6	0.43	0.76	426	
FMET/8 (M-06)	0.28	0.33	398	
FMET/9	1.55	1.22	410	
FMET/10	2.16	2.33	394	
FMET/11 (fluopicolide)	79.97	81.56	382	
Detected by LC/MS but was not detected in the profiles by radio-HPLC.			362	
Detected by LC/MS but was not detected in the profiles by radio-HPLC.			414	
Total	85.40	86.38		

The investigations by LC/MS and LC-MS/MS permitted a total of 99.54% of the radioactivity eliminated in the faeces over the 0 – 48 h period post-dose by the male rats to be assigned a proposed structure which accounted for 85.79% of the administered dose. For the females, a total of 98.96% of the radioactivity eliminated in the faeces over the 0 – 48 h period post-dose was assigned to a proposed structure which accounted for 86.38% of the administered dose.

III. Conclusions

The metabolism of fluopicolide in the rat was investigated following the administration, by oral gavage, of [phenyl U-^{14}C]-fluopicolide at a nominal dose of 100 mg/kg bw (Single Oral High Dose).

- In total, in the male rats, 92.85% of the administered dose was excreted over the 168 h period following dosing whilst in female rats, 94.86% of the administered dose was excreted. The excretion was fast and essentially complete 24 – 48 h post-administration.
- In total, up to 46 radioactive components were detected in the urine samples and up to 14 radioactive components were detected in the faecal extracts, thus indicating that [phenyl U-^{14}C]-fluopicolide was extensively metabolised in the rat following oral dosing.
- Pooled samples of urine over the intervals 0 – 6 h, 6 – 24 h and 24 – 48 h contained up to 33 fractions by radio-HPLC in the male rats and up to 42 fractions in the female rats. Qualitatively, the metabolite profiles were similar between the sexes although a greater number of metabolites were observed in the female urine compared to the male urine and some quantitative differences were also observed.
- Pooled samples of faeces over the intervals 0 – 24 h and 24 – 48 h contained up to 11 fractions by radio-HPLC in the male and female and the metabolite profiles were similar between the sexes.
- 40.76% of the radioactivity eliminated in urine by the male rats and 76.95% by the female rats was able to be assigned to proposed structures which accounted for 1.80% and 4.41% of the administered dose, respectively. 99.54% of the radioactivity eliminated in faeces by the male rats and 98.96% by the female rats was able to be assigned to proposed structures which accounted for 85.40% and 86.38% of the administered dose, respectively.
- No parent fluopicolide was identified in the urine whilst 79.97% and 81.56% of the administered dose in the male and female rats respectively was recovered from the faeces as intact parent molecule.
- Most of the proposed metabolites contained both aromatic rings suggesting that there is not significant cleavage between the rings. Most metabolites were derived from the parent molecule by hydroxylation in the phenyl ring followed by sulphate or glucuronide conjugation and/or by substitution of a chlorine atom in the phenyl ring by glutathione followed by subsequent degradation to the cysteine conjugate. No significant metabolism in the pyridyl ring was observed.
- Overall, a total of 87.2% of the administered radioactivity was assigned a structure for the males and 90.8% of the administered radioactivity for the females and a metabolic pathway is proposed for [phenyl U-^{14}C]-fluopicolide in the rat.

Figure 5.1.1-5: Proposed metabolic pathway for fluopicolide in the rat based upon the results from the Single Oral High Dose [Phenyl-U-^{14}C]-fluopicolide study

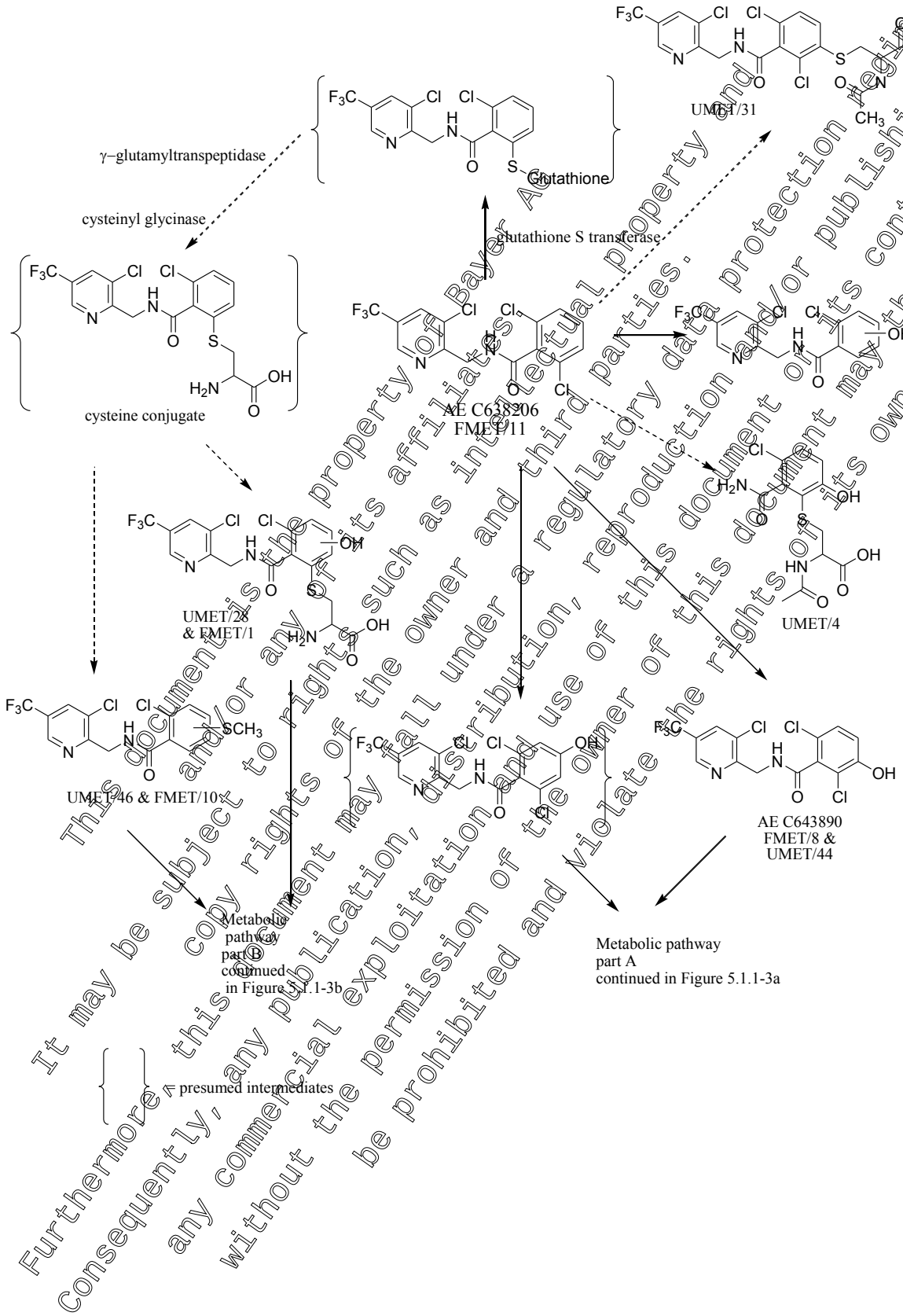


Figure 5.1.1-6: Proposed metabolic pathway for fluopicolide in the rat (continued)

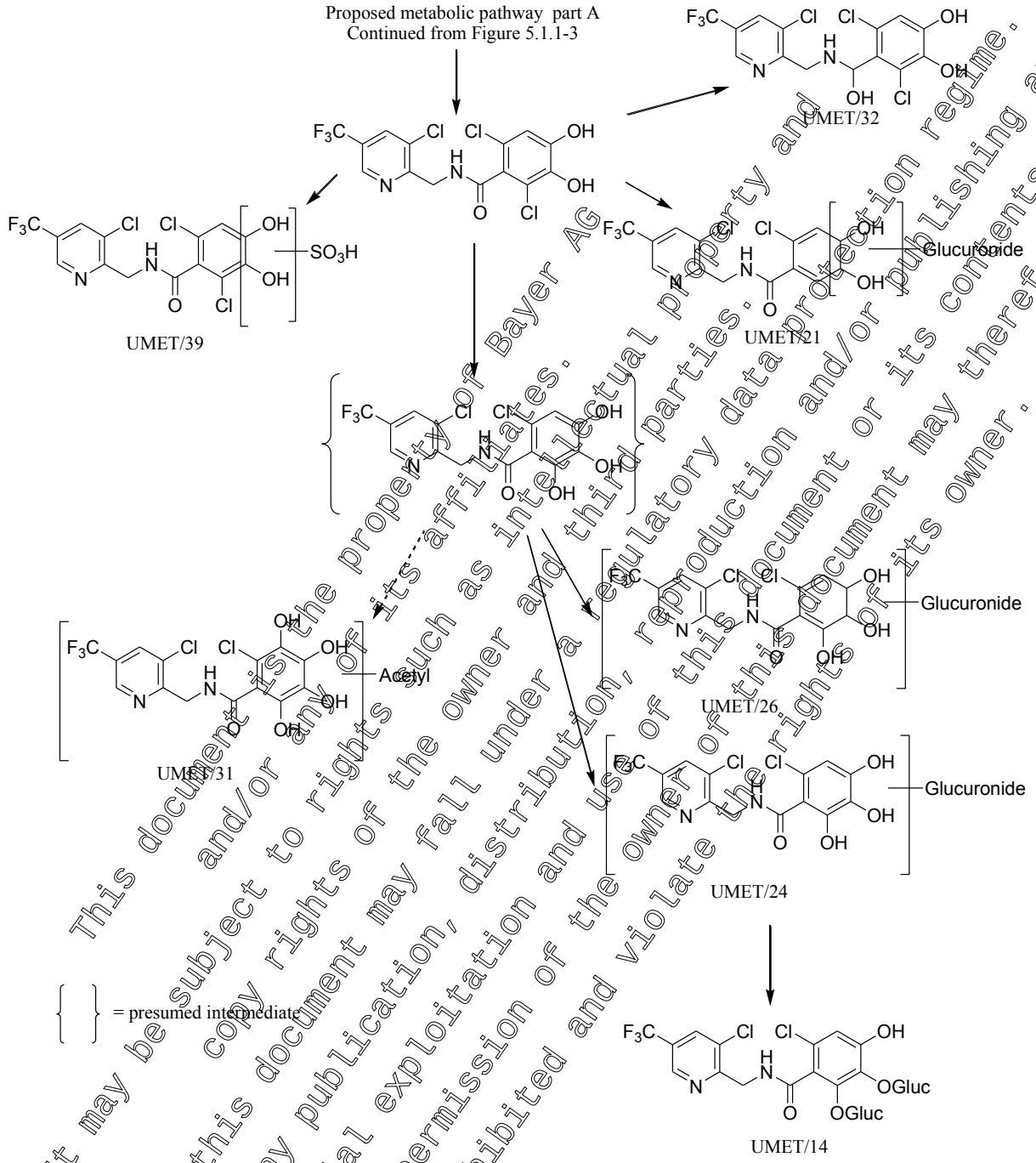
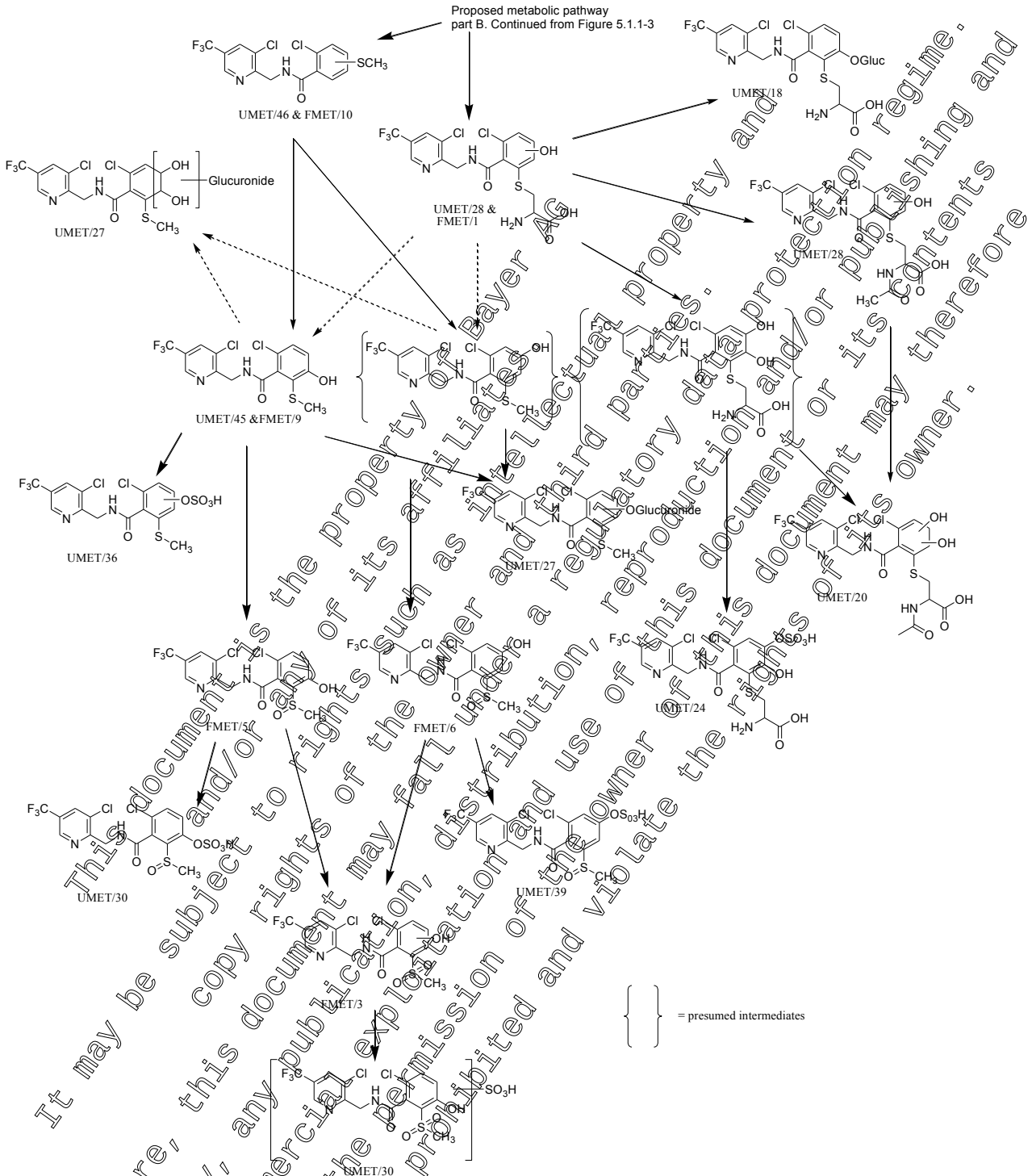


Figure 5.1.1-7: Proposed metabolic pathway for fluopicolide in the rat (continued)



Assessment and conclusion by applicant:

The study is valid and acceptable to investigate the metabolism of [phenyl-U-^{14}C]-fluopicolide in the rat.

Data Point:	KCA 5.1.1/11
Report Author:	[redacted]
Report Year:	2004
Report Title:	[Phenyl-U-14C]-AE C638206: Rat metabolism following administration of a single oral low dose
Report No:	C039583
Document No:	M-227026-02-1
Guideline(s) followed in study:	EU (=EEC): 87/302/EEC, Part B; JMAF: Nousan No. 8147, 24 NOV 2000; USEPA (=EPA): OPPTS 870.7485
Deviations from current test guideline:	not specified
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary:

The metabolism of fluopicolide in the rat was investigated with radiolabelled fluopicolide ([phenyl U-^{14}C]-fluopicolide).

A single oral gavage dose of [phenyl U-^{14}C]-fluopicolide was administered to 4 male and 4 female Sprague Dawley rats at a nominal dose of 10 mg/kg bw (Single Oral Low Dose). Samples of urine were collected at 0 – 6 h, 6 – 24 h and then at 24-hourly intervals up to 168 h and samples of faeces were collected at 24-hourly intervals up to 168 h. These urine and faeces samples were obtained from the rat ADE study Totis, M. (2001), M-204781-01-1 reported at KCA 5.1.1/02.

The radioactivity in pooled samples of urine (0 – 6 h, 6 – 24 h and 24 – 48 h and 48 – 72 h) and faeces (0 – 24 h, 24 – 48 h and 48 – 72 h (males only)) from the male and female rats was characterised by radio-HPLC, and by LC-MS and LC-MS/MS. In total, up to 49 radioactive components were detected in the urine samples and up to 47 radioactive components were detected in the faecal extracts, thus indicating that [phenyl U-^{14}C]-fluopicolide was extensively metabolised in the rat following oral dosing.

In the urine samples, up to 47 different radioactive fractions were observed in the urine of the male rats and up to 49 different radioactive fractions were observed in the urine of the female rats. Qualitatively, the metabolite profiles were similar between the sexes although a greater number of metabolites were observed in the female urine compared to the male urine. In the male rats, the most significant components were UMET/4 and UMET/39 which accounted for 2.01% and 1.21% of the administered dose, respectively; UMET/39 was able to proposed to be M-19 (derived by hydroxylation in the phenyl ring and substitution of one of chlorine atom by a sulphate group). Other components individually amounted to less than 0.60% of the dose and structural assignments indication hydroxylation of the phenyl ring and the subsequent formation of sulphate conjugates, with or without substitution of a chlorine atom of the phenyl ring by glutathione and subsequent degradation to the cysteine conjugate. In the female rats, the most significant components were UMET/33 (1.53% dose), UMET/38 (1.02% dose), UMET/40 (1.32% dose) and UMET/43 (2.31% dose). Structures were assigned for all four and these involved hydroxylation and conjugation of the phenyl ring. Other components individually amounted to less than 0.64% of the dose and structural assignments indicated a similar pattern of metabolism as in male rats.

In the faeces samples, up to 40 different fractions were observed in the faeces of the male rats and up to 47 different fractions were observed in the faeces of the female rats. Qualitatively, the metabolite profiles were similar between the sexes although a greater number of metabolites were observed in the female urine compared to the male urine. In the male rats, the most significant component was FMET/44 which accounted for 39.60% of the dose. Other prominent fractions were FMET/40 which accounted for 5.41% of the dose and FMET/42 which accounted for 10.46% of the dose. FMET/40 was proposed

to be AE 0717560 (hydroxylated parent molecule with a methyl sulphide group replacing one of the chlorine atoms in the phenyl ring) whilst FMET/42 was proposed to have a similar structure to FMET/40 but without the hydroxylation. Other less significant components amounting to up 2.92% of the dose were able to be assigned to similar structures resulting from hydroxylation in the phenyl ring and sulphate conjugation. In the females, parent fluopicolide was again the major component, amounting to 40.91% of the dose and FMET/42 was also significant component was FEMT/42. Other less significant components amounting to up 3.62% of the dose were able to be assigned to similar structures resulting from hydroxylation in the phenyl ring and sulphate conjugation.

Sulphate and glucuronide conjugation was able to be confirmed for a number of the components in urine samples following enzymatic hydrolysis investigations.

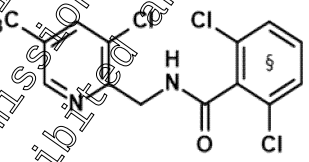
52.40% of the radioactivity eliminated in urine by the male rats and 80.31% by the female rats was able to be assigned to proposed structures which accounted for 4.95% and 9.85% of the administered dose, respectively. 87.25% of the radioactivity eliminated in faeces by the male rats and 79.50% by the female rats was able to be assigned to proposed structures which accounted for 71.91% and 64.64% of the administered dose, respectively. Overall, a total of 76.86% of the administered radioactivity was assigned a structure for the males and 74.49% of the administered radioactivity for the females

Most of the proposed metabolites contained both aromatic rings suggesting that there is not significant cleavage between the rings. Most metabolites were derived from the parent molecule by hydroxylation then conjugation in the phenyl ring and/or by substitution of a chlorine atom in the phenyl ring by glutathione followed by subsequent degradation to the cysteine conjugate. No significant metabolism in the pyridyl ring was observed. A metabolic pathway for fluopicolide in the rat is proposed.

I. Materials and methods

A. Materials

1. Test Material:

IUPAC Name	2,6-dichloro-N-[[3-chloro-5-(trifluoromethyl)-2-pyridinyl]methyl]-benzamide
CAS Name	benzamide, 2,6-dichloro-N-[[3-chloro-5-(trifluoromethyl)-2-pyridinyl]methyl]
Code name	AE C638206
Common name	Fluopicolide
Empirical formula	$C_{14}H_8Cl_3F_3N_2O$
CAS Number	239110-15-7
Molar mass	383.59 g/mol
Chemical structure	 * denotes position of label
Radiolabelled test material	[phenyl-U-^{14}C]-fluopicolide*
Lot number	901-CU-2
Radiochemical purity	> 99% (by HPLC)
Specific radioactivity	59.5 mCi/mmole or 2.202 GBq/mmole
Specific radioactivity of dose suspensions	Checked by HPLC and radio assay
Stability of test compound	Stable at -20˚C
Non-radiolabelled test material	Fluopicolide
Lot number	R001737
Purity	99.3%

*referred to as [phenyl-U-14C]-AE C638206 in the report

2. Vehicle: Aqueous methyl cellulose (0.75% w/w)

3. Test animals:

Species:	Rat
Strain:	Sprague Dawley, CD
Age:	Not stated
Weight at dosing:	151 – 175g
Source:	[redacted]
Acclimation period:	7 days
Identification:	Ear tattoo
Diet:	Certified rodent diet M20 (Pietrement, Provins, France)
Water:	Provided ad libitum (filtered, softened water from municipal supply)
Housing:	Housed in metal wire-mesh bottomed cages in metal trays containing absorbent paper during acclimatisation and in specialised metabolism cages thereafter
Environmental conditions	
Temperature:	22 ± 2˚C
Humidity:	55 ± 15%
Photoperiod:	12 hours
Air changes	Average 15 changes/hour

4. Preparation of dosing solutions

The doses were prepared by mixing weighed amounts of radiolabelled and non-radiolabelled material in acetonitrile to achieve a homogenous solution at the desired specific radioactivity. The solution was then gently evaporated under a stream of nitrogen, appropriate weights of aqueous methyl cellulose (0.75% w/w) added and the resulting solution ground to produce a suspension. The dose suspension was of sufficient quantity and concentration to provide a dose of 1g per 200 g rat body weight. The dose suspensions for the low and high dose groups were prepared on the day of dosing and assayed by HPLC for active ingredient content and for radioactivity content (by LSC) before, during and following the dosing procedure.

B. Study design and methods

1. In life dates: 12th September 2000 to 19th April 2001 (in-life phase); 29th November 2000 – 11th April 2002 (analytical phase).

2. Dose regimen and design of tests

Administered single dose of [phenyl-U-^{14}C]-fluopicolide, route (experiment)	Number of rats and sex	Collection of samples during the test and at sacrifice	Duration
10 mg/kg bw, oral (single high dose)	4 males 4 females	Urine, faeces, cage wash, blood, plasma, organs, tissues, carcass	7 days

The rats each received a single dose of [phenyl-U-^{14}C]-fluopicolide at a target dose of 10 mg/kg bw/d. Urine and faeces samples were collected at intervals until sacrifice at 168-hours, when the blood, organs, tissues and carcass were collected.

3. Dosing

Adequate volumes of the suspension of the test compound in aqueous methyl cellulose (0.75% w/w) were administered to fasted (18-hours) rats via a single oral gavage dose. The doses for each individual animal were calculated based on the weights of the animal prior to dosing (food was replaced one-hour post dose and remained available ad libitum throughout the remainder of the study).

4. Collection of excreta

Following administration of the radiolabelled test substance, animals were kept in specialised individual metabolism units (Jencon's metabowls Mk. III), which permitted the separate quantitative collection of urine and faeces, for a maximum of 168-hours following dosing. Urine was collected at 0-6 and 6-24 hours and subsequently at 24-hour intervals following dosing, whilst faeces was collected at 24-hour intervals following dosing. Cages were washed with distilled water for each 24-hour period post-dose and then with acetonitrile at the end of the in-life phase; cage washes were retained for analysis. Both urine and faeces were collected frozen over solid CO_2 in weighed polystyrene boxes; carded cotton protected the samples from light. Note, these samples were obtained from the rat ADE study Totis, M. (2001), M-204781-01-1 reported at KCA 5.1.1/02.

5. Sacrifice

All animals were killed by exsanguination under Imogene 300 anaesthesia, at 7 days following dose administration.

6. Radioassay

Pooled samples of urine, cage washes and aqueous homogenates of faeces (50% w/v) from the male and female groups were radio assayed by liquid scintillation counting (LSC) in Ultima Gold liquid scintillation cocktail (10mL) after stabilising to ambient temperature and reduced light intensity.

7. Preparation of pooled samples

Aliquots of the 0 – 6 h, 6 – 24 h, 24 – 48 h and 48 – 72 h pooled urine samples from the male and female groups were concentrated under a stream of nitrogen gas, a small volume of methanol was added and the samples were then centrifuged. The resulting supernatants were radio assayed by LSC to determine that they were sufficiently concentrated for adequate chromatography (50,000 – 80,000 dpm in less than 200 µL).

Aliquots of the 0 – 24 h, 24 – 48 h and 48 – 72 h (male only) pooled faeces samples from the male and female groups were extracted with acetonitrile using an Ultra Turrax homogeniser and then centrifuged. The resulting pellet was further extracted with acetonitrile, acetonitrile: water (70:30 v/v), chloroform, acetonitrile: water (80:20 v/v, pH 11) and then finally acetonitrile: water (60:40 v/v, pH 4) using an ultrasonic bath and centrifugation at each step. The combined supernatants were concentrated under a stream of nitrogen gas and radio assayed by LSC.

8. Characterisation

The quantitative profiles of the urine samples and of the faeces extracts were obtained using radio-HPLC. This method comprised of a Phenomenex LUNA C18 (5 µm) 250 × 4.6 mm column eluted with a gradient of 95% A:5% B to 55% A:45% B over 80 min where mobile phase A comprised 1 mM ammonium acetate/0.1% tetrahydrofuran (pH 5.5) and mobile phase B comprised acetonitrile/0.5% tetrahydrofuran. Detection was by UV absorption at 254 nm and with a flow-through radioactivity detector with a 400 µL lithium glass flow cell.

Identification of parent fluopicolide and the metabolites M-06 (AE C643890), M-02 (AE C657188), M-01 (AE C653711), 2-chlorobenzamide, RPA 224241, AE C643805, AE C416656 and M-03 (AE C060800) was attempted by retention time matching to certified reference standards using the radio-HPLC system.

Further characterisation and identification were performed by LC-MS and LC-MS/MS. This method comprised of a Phenomenex Luna C18 (5 µm) 250 × 4.6 mm column eluted with a gradient of 95% A:5% B to 45% A:55% B over 70 min where mobile phase A comprised 10 mM ammonium acetate/0.1% tetrahydrofuran, pH 5.5 and mobile phase B comprised acetonitrile/0.1% tetrahydrofuran or with a gradient of 95% A:5% B to 55% A:45% B over 70 min where mobile phase A comprised 0.05% aqueous trifluoroacetic acid and mobile phase B comprised acetonitrile. Detection was by UV absorption using a DAD, a flow-through radioactivity detector with a 163.2 µL lithium glass flow cell and by ESI mass spectrometry in the positive and negative ion modes. Structures were proposed on the basis of identified molecular ions and fragmentation patterns.

9. Enzymatic hydrolysis

Enzymatic deconjugation experiments were performed with β-Glucuronidase (type B1 from Bovine liver or *E. coli*) and sulfatase (type H1 from *Helix Pomatia*) in order to investigate for the presence of glucuronide and sulphate conjugates. Aliquots of pooled urine from males and females (6 – 24 h) were incubated with enzyme in 0.2 M sodium acetate buffer (pH 5 or pH 6.8) for 18 hours at 37°C. The reactions were quenched by the addition of methanol. The samples were then centrifuged, concentrated, and analysed by radio-HPLC.

II. Results and Discussion

A. Recovery

In males and females, the majority of the elimination of [phenyl-U-^{14}C]-fluopicolide was via the faeces, with a corresponding lower amount being excreted via the urine (see table below).

Table 5.1.1- 46: Excretion of radioactivity at time intervals, expressed as % dose administered

Dose, route	10 mg/kg bw		10 mg/kg bw	
Experiment	single low dose		single low dose	
Duration, sex	168 h, 4 males		168 h, 4 females	
Time period (h)	Mean	SD	Mean	SD
Radioactivity in faeces (% of dose administered, cumulative)				
0-24	53.91	24.64	72.54	7.78
0-48	76.76	7.06	81.30	2.58
0-72	80.88	4.58	81.82	2.69
0-96	82.02	4.02	81.95	2.67
0-120	82.36	3.88	82.02	2.68
0-144	82.50	3.85	82.06	2.68
0-168	82.58	3.83	82.09	2.68
Radioactivity in urine (% of dose administered, cumulative)				
0-6	0.83	0.53	2.10	0.37
0-24	4.85	1.32	9.35	1.06
0-48	8.36	2.91	12.29	3.02
0-72	9.45	3.41	12.72	3.26
0-76	9.89	3.59	12.88	3.30
0-120	10.09	3.67	12.97	3.32
0-144	10.21	3.71	13.04	3.33
0-168	10.30	3.73	13.09	3.34

In total, in the male rats, 92.88% of the administered dose was excreted over the 168 h period following dosing whilst in female rats, 95.18% of the administered dose was excreted. The excretion was essentially complete 72 h post-administration. As such, only samples of urine over the intervals 0 – 6 h, 6 – 24 h, 24 – 48 h and 48 – 72 h and samples of faeces over the intervals 0 – 24 h, 24 – 48 h and 48 – 72 h (males only) were selected for characterisation and identification.

The mean recovery of the sample work-up of the urine samples was 119% whilst the faeces extraction procedure recovered 96.0 – 116.2% of the radioactivity for characterisation.

B. Metabolite quantification

1. Urine

Up to 47 different radioactive fractions were observed in the urine of the male rats and 49 different radioactive fractions were observed in the urine of the female rats. Qualitatively, the metabolite profiles were similar between the sexes although a greater number of metabolites were observed in the female urine.

Peaks in the profiles of the urine samples were sequentially named UMET/#, based upon their respective elution times. In the male rats, the most significant components were UMET/4 which accounted for 2.01% of the dose and UMET/39 which accounted for 1.21% of the dose. Ten fractions each individually amounted to 0.32 – 0.60% of the dose (UMET/2, UMET/5, UMET/6, UMET/13, UMET/35, UMET/36, UMET/38, UMET/43, UMET/46 and UMET/51); a further six fractions were found to account for 0.11 – 0.28% of the dose (UMET/3, UMET/10, UMET/11, UMET/15, UMET/30, and UMET/53); and the remaining 29 detectable fractions each accounted for 0.006 – 0.074% of the dose. In the female rats, the most significant components were UMET/43 (2.31% of the dose), UMET/33 (1.53% of the dose), UMET/38 (1.02% of the dose) and UMET/40 (1.32% of the dose). Four fractions each individually amounted to 0.41 – 0.64% of the dose (UMET/29, UMET/32, UMET/34 and UMET/36); a further four fractions amounted to 0.22 – 0.38% of the dose (UMET/3, UMET/4, UMET/24, UMET/24, UMET/31, UMET/45, UMET/46, UMET/51 and UMET/53); and the remaining 32 detectable fractions each accounted for 0.0001 – 0.16% of the dose. The table below presents the quantification data for the metabolites in the urine from the male and female rats.

Table 5.1.1- 47: Quantification of metabolites in urine from male and female rats following a single oral dose of [phenyl-U-^{14}C]-fluopicolide at the rate of 10 mg/kg b/w (results are expressed in terms of percentage of administered radioactivity)

Metabolite ID	Males (% dose)					Female (% dose)				
	0-6 h	6-24 h	24-48 h	48-72 h	Sum	0-6 h	6-24 h	24-48 h	48-72 h	Sum
UMET/1	0.009	n.d.	n.d.	n.d.	0.009	0.021	0.039	0.036	n.d.	0.096
UMET/2	0.049	0.296	0.133	0.028	0.506	0.005	0.047	0.020	0.011	0.083
UMET/3	0.008	0.115	0.048	0.029	0.200	0.043	0.115	0.061	0.020	0.239
UMET/4	0.035	1.058	0.679	0.255	2.007	0.015	0.165	0.055	0.033	0.269
UMET/5	0.038	0.334	0.196	0.013	0.581	0.047	n.d.	0.035	0.001	0.083
UMET/6	0.303	0.014	n.d.	0.003	0.320	0.044	n.d.	0.005	n.d.	0.048
UMET/7	n.d.	n.d.	0.006	n.d.	0.006	0.020	0.077	n.d.	n.d.	0.096
UMET/8	n.d.	0.023	n.d.	n.d.	0.023	0.003	n.d.	n.d.	n.d.	0.003
UMET/9	0.007	n.d.	n.d.	0.002	0.009	n.d.	0.006	0.008	0.001	0.015
UMET/10	0.008	0.004	0.082	0.011	0.106	n.d.	0.013	0.020	0.001	0.034
UMET/11	n.d.	0.154	0.056	0.005	0.215	n.d.	n.d.	0.013	n.d.	0.013
UMET/12	n.d.	0.011	0.018	0.000	0.029	n.d.	n.d.	0.021	n.d.	0.021
UMET/13	n.d.	0.164	0.154	0.018	0.336	n.d.	0.112	0.033	n.d.	0.145
UMET/14	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.038	0.028	0.030	0.096
UMET/15	0.021	0.127	0.092	0.036	0.276	0.022	0.029	0.019	0.007	0.078
UMET/16	0.001	n.d.	0.009	0.021	0.031	0.006	n.d.	n.d.	n.d.	0.006
UMET/17	n.d.	0.017	0.004	0.001	0.023	0.020	n.d.	n.d.	n.d.	0.020
UMET/18	n.d.	n.d.	n.d.	n.d.	n.d.	0.016	0.018	0.001	0.001	0.035
UMET/19	n.d.	n.d.	0.011	n.d.	0.011	0.040	0.025	0.007	n.d.	0.072
UMET/20	n.d.	0.018	0.012	0.003	0.033	n.d.	n.d.	n.d.	n.d.	n.d.
UMET/21	0.002	0.068	0.004	n.d.	0.074	0.032	0.035	0.010	0.002	0.079
UMET/22	0.006	n.d.	n.d.	n.d.	0.006	0.024	0.078	0.051	0.002	0.155
UMET/23	0.030	0.010	0.003	0.005	0.047	n.d.	n.d.	n.d.	n.d.	n.d.
UMET/24	0.004	0.015	n.d.	0.009	0.027	0.068	0.111	0.192	n.d.	0.371
UMET/25	0.026	0.010	0.020	0.013	0.068	0.065	0.113	0.077	0.001	0.256
UMET/26	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.012	n.d.	n.d.	0.012
UMET/27	0.002	0.010	0.032	n.d.	0.044	n.d.	0.018	n.d.	0.001	0.019
UMET/28	n.d.	0.037	n.d.	0.004	0.040	0.025	0.046	0.044	n.d.	0.116

Metabolite ID	Males (% dose)					Female (% dose)				
	0-6 h	6-24 h	24-48 h	48-72 h	Sum	0-6 h	6-24 h	24-48 h	48-72 h	Sum
UMET/29	n.d.	0.007	0.020	0.002	0.030	0.067	0.574	n.d.	0.001	0.642
UMET/30	n.d.	0.123	n.d.	n.d.	0.123	n.d.	n.d.	n.d.	n.d.	n.d.
UMET/31	n.d.	n.d.	n.d.	n.d.	n.d.	0.226	0.036	n.d.	0.001	0.264
UMET/32	0.010	n.d.	0.020	n.d.	0.030	0.053	0.349	0.113	0.005	0.520
UMET/33	n.d.	0.031	0.030	n.d.	0.060	0.305	1.211	n.d.	0.009	1.525
UMET/34	0.017	0.016	0.019	n.d.	0.051	0.039	n.d.	0.359	0.010	0.407
UMET/35	0.002	0.007	0.537	0.054	0.600	0.023	0.005	n.d.	0.012	0.040
UMET/36	n.d.	0.304	n.d.	0.066	0.370	0.065	0.512	n.d.	0.011	0.588
UMET/37	0.010	n.d.	n.d.	n.d.	0.010	0.027	0.052	0.008	0.004	0.091
UMET/38	0.146	0.276	0.041	n.d.	0.463	0.400	0.570	0.042	0.007	1.018
UMET/39	0.018	0.222	0.600	0.368	1.208	n.d.	n.d.	n.d.	n.d.	n.d.
UMET/40	n.d.	n.d.	n.d.	n.d.	n.d.	0.077	0.040	1.200	0.001	1.318
UMET/41	n.d.	n.d.	0.027	n.d.	0.027	0.044	0.044	n.d.	0.037	0.125
UMET/42	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.0001	0.0001
UMET/43	n.d.	0.150	0.188	0.021	0.359	0.098	2.182	0.027	0.001	2.307
UMET/44	n.d.	0.023	0.008	n.d.	0.031	n.d.	n.d.	n.d.	n.d.	n.d.
UMET/45	0.003	n.d.	n.d.	0.009	0.012	0.048	0.014	0.154	0.002	0.218
UMET/46	n.d.	0.109	0.164	0.046	0.320	0.047	0.207	0.003	0.002	0.260
UMET/47	0.005	0.014	0.006	0.008	0.033	n.d.	n.d.	n.d.	n.d.	n.d.
UMET/48	0.001	0.005	0.024	0.003	0.033	n.d.	0.009	0.021	0.01	0.040
UMET/49	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.003	0.01	0.013
UMET/50	0.001	0.021	0.020	0.003	0.045	n.d.	0.018	n.d.	0.063	0.081
UMET/51	0.066	0.181	0.181	0.033	0.461	0.029	0.165	0.116	0.069	0.379
UMET/52	n.d.	0.004	n.d.	0.004	0.008	n.d.	n.d.	0.003	0.013	0.016
UMET/53	0.004	0.041	0.066	0.019	0.130	n.d.	0.129	0.144	0.014	0.287
UMET/54	0.020	n.d.	n.d.	0.002	0.022	0.025	0.035	0.013	0.011	0.084
UMET/55	n.d.	n.d.	n.d.	n.d.	n.d.	0.008	n.d.	0.006	0.023	0.037
Total	0.834	4.018	3.568	1.092	9.452	2.096	7.251	2.945	0.429	12.72

n.d. = not detected, n.a. = not applicable.

2. Faeces

Up to 40 different fractions were observed in the faeces of the male rats and up to 47 different fractions were detected in the faeces of the female rats. Qualitatively, the metabolite profiles were similar between the sexes although a greater number of metabolites were observed in the female faeces.

Peaks in the profiles of the faeces samples were sequentially named FMET/#, based upon their respective elution times. In the male rats, the major component was FMET/44 which accounted for 39.60% of the dose. Other prominent fractions were FMET/40 which accounted for 5.41% of the dose and FMET/42 which accounted for 10.46% of the dose. Eleven components accounted for 1.09% to 2.92% of the dose (FMET/16, FMET/18, FMET/20, FMET/22, FMET/26, FMET/31, FMET/32, FMET/36, FMET/38, FMET/45 and FMET/46), whilst the remaining 26 fractions with detectable radioactivity represented between 0.01% and 0.99% of the administered radioactivity. In the female rats, the major component was also FMET/44 which accounted for 40.91% of the dose. The other prominent fraction was FMET/42 which accounted for 8.17% of the dose. Eleven components accounted for 1.07% to 3.62% of the dose (FMET/14 FMET/16, FMET/17, FMET/18, FMET/20, FMET/22, FMET/30, FMET/32, FMET/38, FMET/40 and FMET/45) whilst the remaining fractions with detectable radioactivity represented between 0.02% and 0.90% of the administered radioactivity. The table below presents the quantification data for the metabolites in the faeces from the male and female rats.

Table 5.1.1- 48: Quantification of metabolites in faeces from male and female rats following a single oral dose of [phenyl-U-^{14}C]-fluopicolide at the rate of 10 mg/kg b/w (results are expressed in terms of percentage of administered radioactivity)

Metabolite ID	Males (%dose)				Females (%dose)		
	0-24 h	24-48 h	48-72 h	Sum	0-24 h	24-48 h	Sum
FMET/1	n.d.	n.d.	0.01	0.01	n.d.	n.d.	n.d.
FMET/2	n.d.	n.d.	n.d.	n.d.	n.d.	0.05	0.05
FMET/3	0.29	0.03	0.02	0.33	0.47	0.02	0.49
FMET/4	n.d.	0.03	0.00	0.04	0.28	0.03	0.31
FMET/5	n.d.	n.d.	n.d.	n.d.	n.d.	0.02	0.02
FMET/6	n.d.	0.07	0.00	0.07	0.50	0.03	0.53
FMET/7	n.d.	0.02	0.01	0.03	0.59	0.03	0.62
FMET/8	n.d.	n.d.	0.01	0.01	n.d.	0.04	0.04
FMET/9	n.d.	n.d.	n.d.	n.d.	n.d.	0.03	0.03
FMET/10	n.d.	n.d.	n.d.	n.d.	0.38	0.04	0.42
FMET/11	n.d.	n.d.	n.d.	n.d.	0.23	0.04	0.27
FMET/12	n.d.	0.05	n.d.	0.05	0.31	0.01	0.32
FMET/13	n.d.	n.d.	0.02	0.02	0.57	0.14	0.72
FMET/14	0.44	0.04	0.02	0.49	0.76	0.31	1.07
FMET/15	n.d.	n.d.	0.02	0.02	0.30	0.00	0.30
FMET/16	2.11	0.78	0.02	2.92	1.60	0.12	1.73
FMET/17	n.d.	0.23	0.04	0.26	1.50	0.23	1.73
FMET/18	1.29	0.21	n.d.	1.50	1.00	0.13	1.13
FMET/19	n.d.	0.17	n.d.	0.17	n.d.	0.19	0.19
FMET/20	0.53	0.51	0.05	1.09	1.59	0.28	1.88
FMET/21	n.d.	0.12	0.02	0.14	0.28	0.00	0.28
FMET/22	1.32	0.27	0.03	1.62	1.63	0.45	2.07
FMET/23	0.40	0.36	0.04	0.80	0.79	0.11	0.90
FMET/24	0.30	0.16	0.03	0.49	0.56	0.04	0.60
FMET/25	0.48	0.12	0.03	0.63	0.54	0.05	0.59
FMET/26	n.d.	0.92	0.59	1.51	0.69	0.07	0.76
FMET/27	0.50	0.26	0.02	0.79	n.d.	0.08	0.08
FMET/28	n.d.	0.18	0.05	0.22	n.d.	n.d.	n.d.
FMET/29	n.d.	0.16	0.13	0.29	n.d.	0.26	0.26
FMET/30	n.d.	n.d.	n.d.	n.d.	1.15	n.d.	1.15
FMET/31	0.51	0.63	0.53	1.66	n.d.	0.82	0.82
FMET/32	0.81	1.44	0.22	2.47	1.47	0.46	1.94
FMET/33	n.d.	0.20	0.03	0.23	0.77	0.11	0.88
FMET/34	n.d.	n.d.	n.d.	n.d.	n.d.	0.03	0.03
FMET/35	0.32	0.14	0.01	0.47	n.d.	0.04	0.04
FMET/36	0.77	0.27	0.04	1.09	0.77	0.10	0.88
FMET/37	n.d.	n.d.	n.d.	n.d.	n.d.	0.04	0.04
FMET/38	1.27	1.29	0.21	2.77	1.86	0.51	2.37
FMET/39	n.d.	n.d.	n.d.	n.d.	0.09	0.04	0.13
FMET/40	2.39	2.72	0.30	5.41	1.81	1.80	3.62
FMET/41	n.d.	n.d.	0.15	0.15	n.d.	n.d.	n.d.
FMET/42	9.21	1.18	0.06	10.46	7.46	0.72	8.17
FMET/43	0.82	0.14	0.02	0.99	0.55	0.09	0.64
FMET/44	39.06	0.51	0.04	39.60	40.29	0.62	40.91
FMET/45	0.93	0.18	n.d.	1.11	1.04	0.15	1.19
FMET/46	0.89	0.21	0.03	1.13	n.d.	0.20	0.20
FMET/47	n.d.	n.d.	n.d.	n.d.	0.67	0.07	0.74
FMET/48	0.75	0.10	n.d.	0.84	n.d.	0.11	0.11
FMET/49	0.47	0.05	n.d.	0.52	n.d.	n.d.	n.d.

Metabolite ID	Males (%dose)				Females (%dose)		
	0-24 h	24-48 h	48-72 h	Sum	0-24 h	24-48 h	Sum
FMET/50	n.d.	n.d.	n.d.	n.d.	n.d.	0.05	0.05
FMET/51	n.d.	n.d.	n.d.	n.d.	n.d.	0.02	0.02
FMET/52	n.d.	n.d.	0.01	0.01	n.d.	n.d.	n.d.
Total	65.86	13.77	2.79	82.42	72.54	8.76	81.31

n.d. = not detected, n.a. = not applicable

C. Metabolite identification

1. Urine

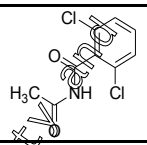
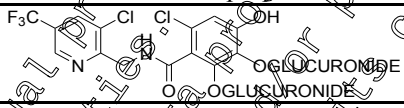
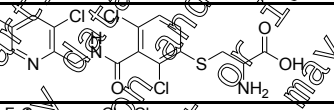
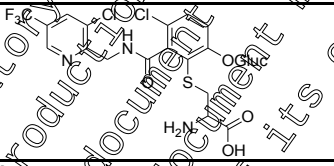
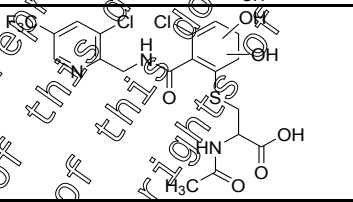
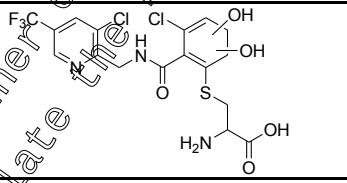
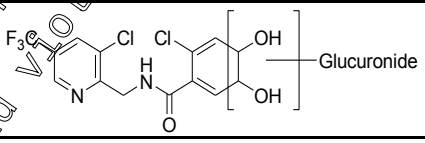
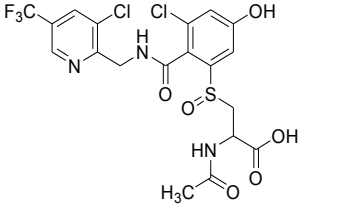
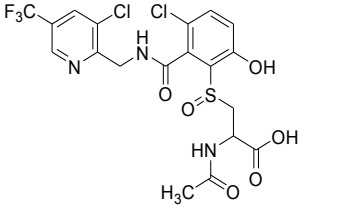
By retention time matching, no parent fluopicolide was observed in the urine samples. UMET/2 had a similar retention time to AE C643805 (3-hydroxy BAM) but subsequent LC-MS indicated that UMET/2 was N-acetylated BAM. UMET/11 had a similar retention time to M-02 (AE C657188) but no structure could be proposed by LC-MS. UMET/15 had a similar retention time to M-01 (AE C653711) but no structure could be proposed by LC-MS beyond confirmation that the metabolite had a mass of 418 Da and contained two chlorine atoms. UMET/21 co-eluted with RPA 224241 but a different structure was supported by LC-MS. UMET/51 co-eluted with M-06 (AE C643890) which was supported by LC-MS. UMET/52 had a similar elution time to M-03 (AE 0608000) but no structure was proposed by LC/MS.

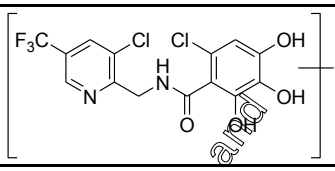
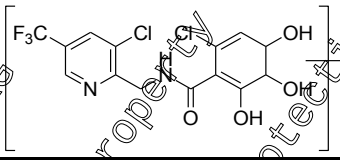
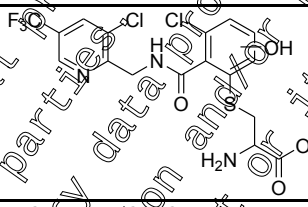
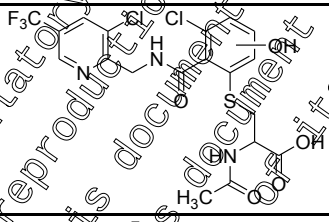
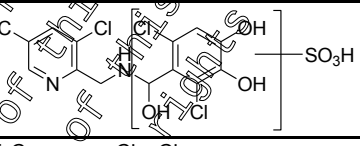
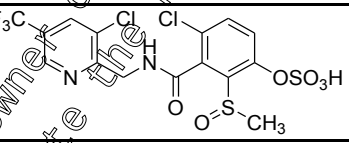
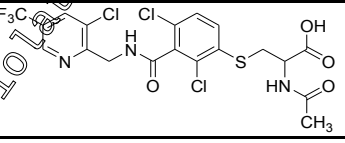
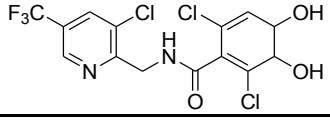
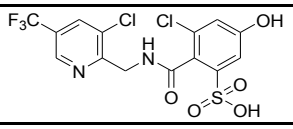
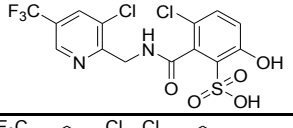
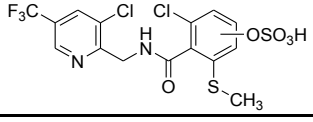
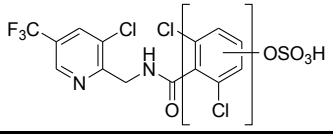
Analysis by LC-MS and LC-MS/MS enabled structures to be proposed for a number of the fractions, this analysis also indicated than some peaks in the profile contained more than one component but since up to 55 different fractions had already been resolved, limited improvement to the profile resolution by altering the chromatography would be expected. The assignments made for the other more significant components in the urine profiles are summarised below:

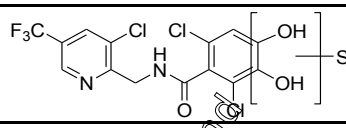
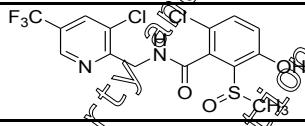
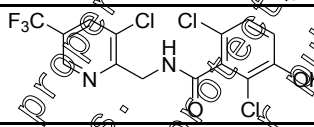
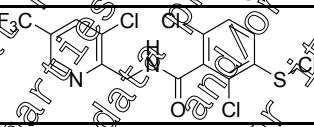
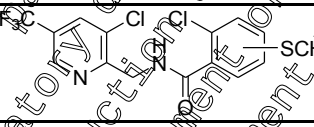
- UMET/39 was assigned to M-19 (dihydroxy sulphate of fluopicolide).
- UMET/35, UMET/38 and UMET/46 were all assigned to structures that involved hydroxylation of the phenyl ring and subsequent formation of sulphate conjugates.
- UMET/36 and UMET/43 were also assigned to structures that involved hydroxylation of the phenyl ring and subsequent formation of sulphate conjugates, but had additionally lost one of the chlorine atoms on the phenyl ring to be replaced with either a methyl sulphide or methyl sulfone group. Both components are likely to have resulted from initial substitution of a chlorine atom on the phenyl ring by glutathione (catalysed by glutathione-S-transferase) followed by degradation to the cysteine conjugate. These conjugates could then be cleaved *via* β-elimination by cysteine conjugate β-lyases leaving a resulting thiol that could serve as the methyl acceptor. A mechanism known as the thiomethyl shunt. The resulting S-methyl could then have been oxidised to provide the proposed sulfone.
- UMET/30 was assigned to a structure that was hydroxylated in the phenyl ring at the 1, 2 and 3 positions (losing one chlorine atom) followed by glucuronide conjugation.
- UMET/53 was proposed as fluopicolide containing a methyl sulphide substation in the phenyl ring.

The overall identification of the metabolites in the urine from the male and female rats is summarised in the table below.

Table 5.1.1- 49: Identification of metabolites in urine from male and female rats following a single oral dose of [Phenyl-U-^{14}C]-fluopicolide at a rate of 10 mg/kg b/w.

Metabolite ID	%dose		Assigned molecular mass	proposed structure
	Males	Females		
UMET/2	0.51	0.08	231	
UMET/8	0.02	0.003	385	No structure proposed
UMET/15	0.28	0.08	418	No structure proposed
UMET/17	0.02	0.02	748	
UMET/19	0.01	0.07	519	
UMET/21	0.07	0.08	629	
UMET/24	0.03	0.37	543	
UMET/25	0.07	0.26	501	
			558	
UMET/26	n.d.	0.01	541	
UMET/27	0.04	0.02	541	
UMET/29	0.03	0.64	578	No structure proposed

Metabolite ID	%dose		Assigned molecular mass	proposed structure
	Males	Females		
UMET/30	0.12	n.d.	572	
UMET/31	n.d.	0.26	574	
UMET/32	0.03	0.52	483	
UMET/33	0.06	1.53	525	
UMET/35	0.60	0.04	496	
UMET/36	0.37	0.59	506	
UMET/37	0.01	0.09	543	
UMET/38	0.46	1.02	416	
UMET/39 (M-19)	1.21	n.d.	444	
UMET/40	n.d.	1.32	444	
UMET/43	0.36	2.30	490	
UMET/45	0.01	0.22	478	

Metabolite ID	%dose		Assigned molecular mass	proposed structure
	Males	Females		
UMET/46	0.32	0.26	494	
UMET/47 (AE 0717559)	0.03	0.04	426	
UMET/51 (M-06 (AE C643890))	0.46	0.38	398	
UMET/53	0.13	0.29	428	
UMET/54	0.02	0.08	394	
Total	5.28	10.58		

n.d. = not detected

The investigations by LC/MS and LC-MS/MS permitted a total of 52.40% of the radioactivity eliminated in the urine over the 0 – 72 h period post-dose by the male rats to be assigned a proposed structure which accounted for 4.95% of the administered dose. For the females, a total of 80.31% of the radioactivity eliminated in the urine over the 0 – 72 h period post-dose was assigned to a proposed structure which accounted for 9.85% of the administered dose.

2. Faeces

By retention time matching, parent fluopicolide was assigned to the principle component FMET/44 and this assignment was confirmed by mass spectroscopy. FMET/3 had a similar retention time to M-01 (AE C653711) but LC-MS led to a proposed structure of N-acetylated BAM. FMET/38 had a similar retention time to M-06 (AE C643890) and this assignment was confirmed by mass spectroscopy.

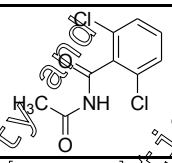
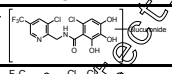
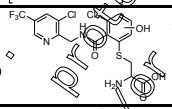
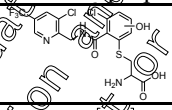
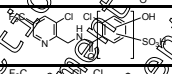
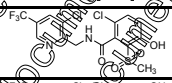
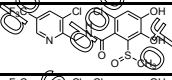
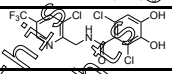
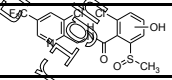
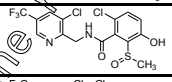
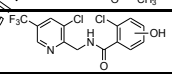
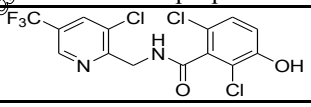
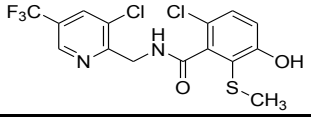
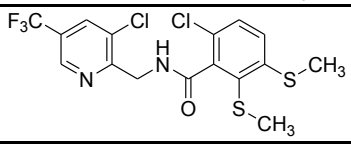
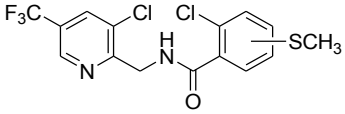
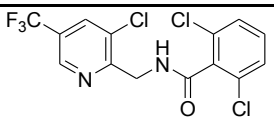
Analysis by LC-MS and LC-MS/MS enabled structures to be proposed for a number of the fractions. The assignments made for the more significant components in the faeces profiles are summarised below:

- FMET/40 was proposed to be AE 0717560 (hydroxylated parent molecule with a methyl sulphide group replacing one of the chlorine atoms in the phenyl ring).
- FMET/42 was proposed to have a similar structure to FMET/40 but without the hydroxylation.
- FMET/16, FMET/26, FMET/31, FMET/32, FMET/36 and FMET38 were proposed to be similar structures resulting from hydroxylation in the phenyl ring and sulphate conjugation.

proposed to be AE 0717560 (hydroxylated); was assigned to a similar non-hydroxylated structure.

The overall identification of the metabolites in the faeces from the male and female rats is summarised in the table below.

Table 5.1.1- 50: Identification of metabolites in faeces from male and female rats following a single oral dose of [phenyl-U-^{14}C]-fluopicolide at a rate of 10 mg/kg b/w.

Metabolite ID	%dose		Molar Mass	Proposed Structure
	Males	Females		
FMET/3 (N-acetylated BAM)	0.33	0.49	406	
FMET/14	0.49	1.07	572	
FMET/16	2.92	1.73	483	
FMET/17	0.26	1.73	513	No structure proposed
FMET/18	1.50	1.13	525	
FMET/23	0.80	0.90	444	
FMET/26 (AE 916598)	1.51	0.76	442	
FMET/27	0.79	0.08	442	
FMET/29	0.29	0.26	414	
FMET/31	1.66	0.82	426	
FMET/32	2.47	1.94	426 AE 0717559	
FMET/36	1.09	0.88	364	
FMET/37	n.d.	0.04	473 or 456	No structure proposed
FMET/38 (M-06 (AE C643890))	2.77	2.37	398	
FMET/40 (AE 0717560)	5.41	3.62	410	
FMET/41	0.15	n.d.	440	
FMET/42	10.46	8.17	394	
FMET/44 (fluopicolide)	39.60	40.91	382	
Total	72.50	66.90		

The investigations by LC/MS and LC-MS/MS permitted a total of 87.25% of the radioactivity eliminated in the faeces over the 0 – 72 h period post-dose by the male rats to be assigned a proposed structure which accounted for 71.91% of the administered dose. For the females, a total of 79.50% of the radioactivity eliminated in the faeces over the 0 – 48 h period post-dose was assigned to a proposed structure which accounted for 64.64% of the administered dose.

2. Enzymatic hydrolysis

The β-Glucuronidase and sulfatase hydrolysis experiments on the urine samples from the male and female rats were difficult to interpret given the high number of resolved fractions in the profiles before hydrolysis (~49 resolved by HPLC). Nevertheless, several components were observed to increase following enzymatic deconjugation. In the male urine samples, UMET/3, UMET/17 and UMET/21 were probably glucuronide conjugates; UMET/4, UMET/35, UMET/38 and UMET/43 were probably sulphate conjugates whilst UMET/20, UMET/34, UMET/41, UMET/42, UMET/45 and UMET/35, UMET/40, UMET/43 and UMET/44 were probably sulphate conjugates whilst UMET/21 appeared to contain both glucuronide and sulphate conjugation.

III. Conclusions

The metabolism of fluopicolide in the rat was investigated following the administration, by oral gavage, of [phenyl U-^{14}C]-fluopicolide at a nominal dose of 10 mg/kg bw (Single Oral Low Dose).

- In total, in the male rats, 92.88% of the administered dose was excreted over the 168 h period following dosing whilst in female rats 95.18% of the administered dose was excreted. Faeces was the main route of elimination and the excretion was fast and essentially complete 48 – 72 h post-administration.
- In total, up to 49 radioactive components were detected in the urine samples and up to 47 radioactive components were detected in the faecal extracts, thus indicating that [phenyl U-^{14}C]-fluopicolide was extensively metabolised in the rat following oral dosing.
- Pooled samples of urine over the intervals 0 – 6 h, 6 – 24 h, 24 – 48 h and 48 – 72 h contained up to 47 fractions by radio-HPLC in the male rats and up to 49 fractions in the female rats. Qualitatively, the metabolite profiles were similar between the sexes although a greater number of metabolites were observed in the female urine compared to the male urine and some quantitative differences were also observed.
- Pooled samples of faeces over the intervals 0 – 24 h, 24 – 28 h and 48 – 72 h (males only) contained up to 40 fractions by radio-HPLC in the male rats and up to 47 fractions in the female rats. Qualitatively, the metabolite profiles were similar between the sexes although a greater number of metabolites were observed in the female faeces compared to the male faeces and some quantitative differences were also observed.
- 52.40% of the radioactivity eliminated in urine by the male rats and 80.31% by the female rats was able to be assigned to proposed structures which accounted for 4.95% and 9.85% of the administered dose, respectively. 87.25% of the radioactivity eliminated in faeces by the male rats and 79.50% by the female rats was able to be assigned to proposed structures which accounted for 71.91% and 64.64% of the administered dose, respectively.
- No parent fluopicolide was identified in the urine whilst 39.60% and 40.91% of the administered dose in the male and female rats respectively was recovered from the faeces as intact parent molecule.

- Most of the proposed metabolites contained both aromatic rings suggesting that there is not significant cleavage between the rings. Most metabolites were derived from the parent molecule by hydroxylation then conjugation in the phenyl ring and/or by substitution of a chlorine atom in the phenyl ring by glutathione followed by subsequent degradation to the cysteine conjugate. No significant metabolism in the pyridyl ring was observed.
- Overall, a total of 76.86% of the administered radioactivity was assigned a structure for the males and 74.49% of the administered radioactivity for the females and a metabolic pathway is proposed for [phenyl U-^{14}C]-fluopicolide in the rat.

Figure 5.1.1-8: Proposed metabolic pathway for fluopicolide in the rat based upon the results from the Single Oral Low Dose [Phenyl-U-^{14}C]-fluopicolide study

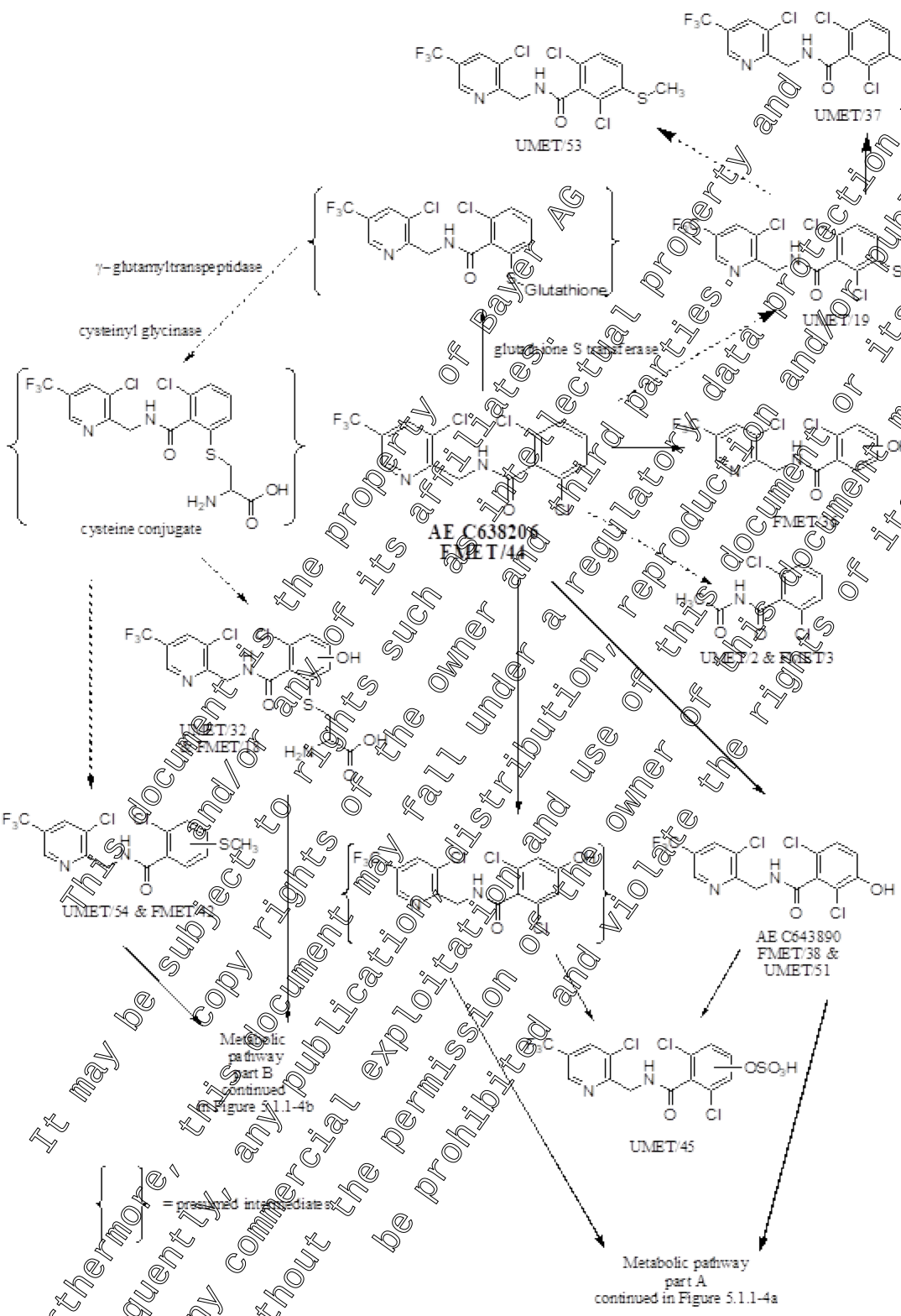


Figure 5.1.1-9: Proposed metabolic pathway for fluopicolide in the rat (continued)

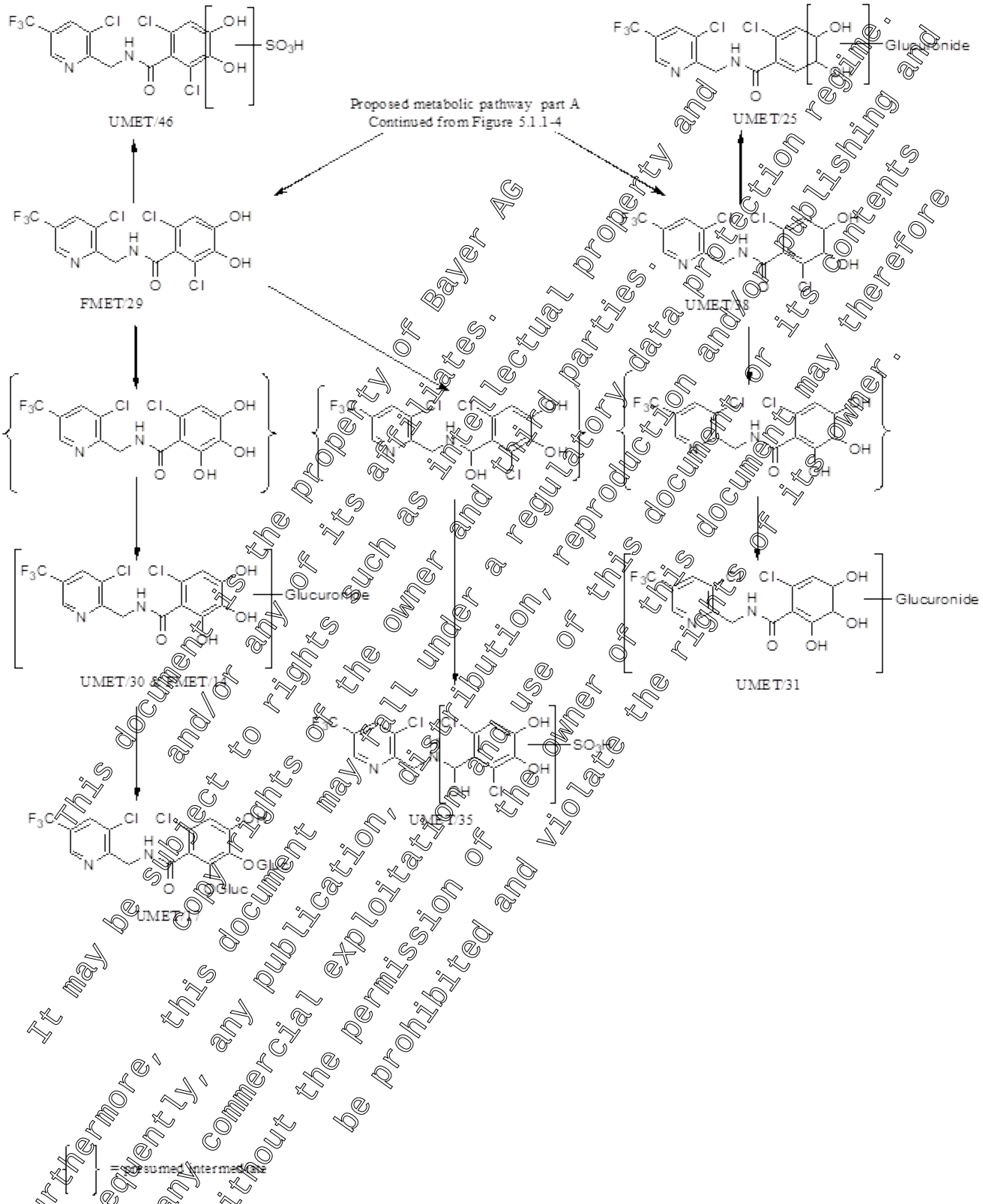
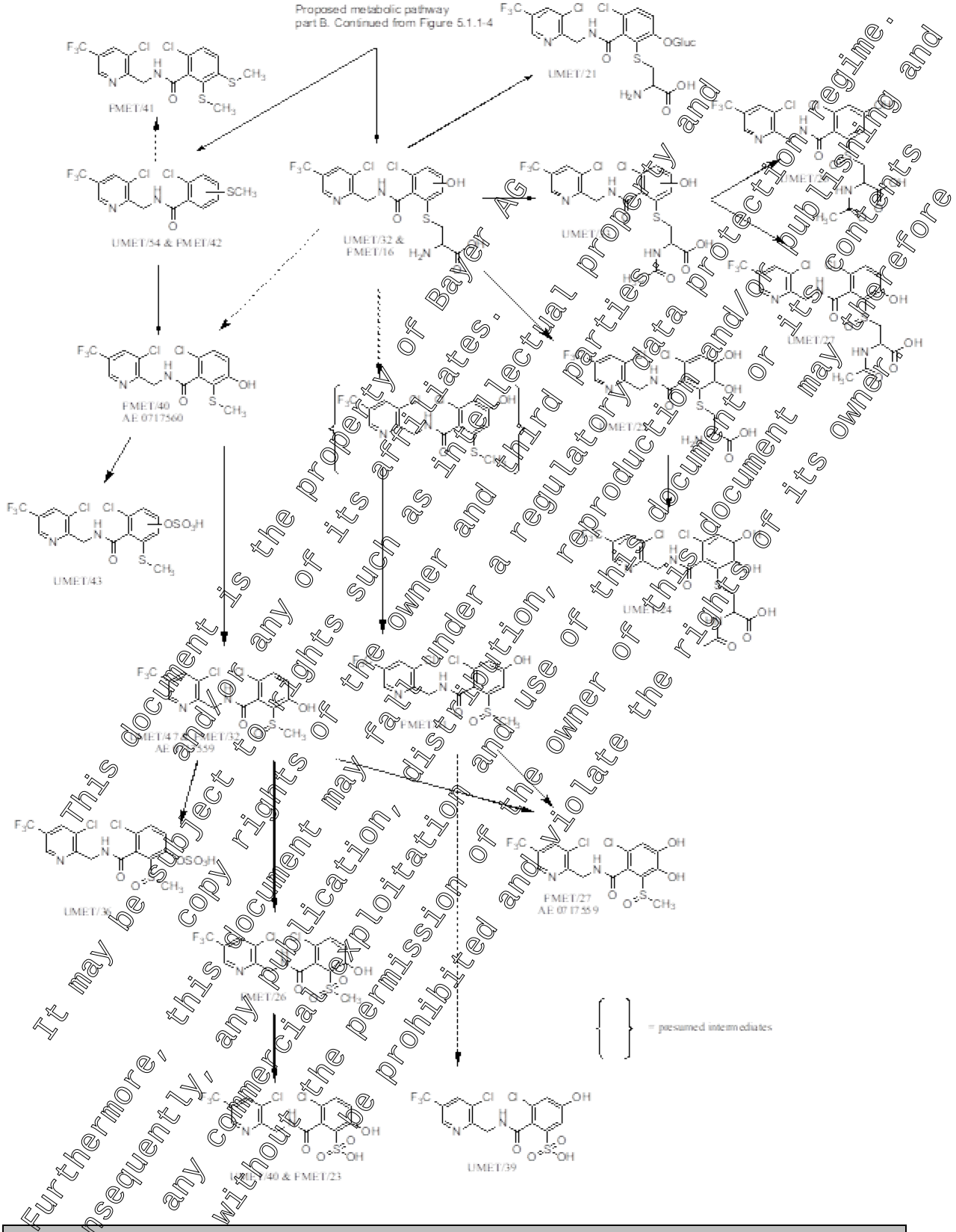


Figure 5.1.1-10: Proposed metabolic pathway for fluopicolide in the rat (continued)



Assessment and conclusion by applicant:

The study is valid and acceptable to investigate the metabolism of [phenyl-U-^{14}C]-fluopicolide in the rat.

Data Point:	KCA 5.1.1/12
Report Author:	[redacted]
Report Year:	2004
Report Title:	[Phenyl-U-14C]-AE C638206: Repeat oral low dose A.D.M.E. study in the rat
Report No:	C039584
Document No:	M-227027-02-1
Guideline(s) followed in study:	EU (=EEC): 87/307/EEC; JMAF: 12, Nousan No 8147, NOV 24, 2000; USEPA (=EPA): OPPTS 870.7485
Deviations from current test guideline:	None
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary:

The absorption, distribution, excretion and metabolism of [phenyl-U-^{14}C]-fluopicolide (referred to as AE C638206 in in the report) was investigated in male and female Sprague Dawley rats following repeated oral dosing at a nominal dose level of 10 mg/kg bw.

[Phenyl-U-^{14}C]-fluopicolide was administered orally to two groups of five male or female rats at a low dose of 10 mg/kg bw/day for 14 days. Urine and faeces samples were collected throughout the dosing period and for a further six days after the final dose until sacrifice, when organs, GIT, skin, and carcass were collected.

Mean recoveries were 95.51±1.19% of the administered dose in male rats and 96.30±0.39% in female rats. Oral absorption after multiple dosing (calculated from the sum of radioactivity in urine, cage washes and tissues) was a minimum of 16.64% in males and *ca.* 23.82% in females. Data from previous bile excretion studies indicate that the actual level of oral bioavailability will be much higher as biliary excretion was found to be a major route of elimination.

The routes and rates of elimination were similar between male and female rats. The main route of elimination was *via* faeces, a mean of 78.863% of the administered dose in male rats and 72.479% in female rats eliminated during the study. The proportion of radioactivity eliminated *via* the urinary route (including cage wash) was 16.346% in male rats and 23.361% in female rats.

Overall, the multiple dosing did not have any significant impact in the absorption, distribution, metabolism, and elimination compared to results after single oral dosing. Levels of radioactivity remaining in tissues at sacrifice represented a sum total of 0.30% of the administered dose in male rats and 0.46% in female rats. The highest mean tissue concentrations of [14C]-fluopicolide equivalents in male tissues were found in the liver (1.356±0.082 µg/equiv./g), followed by the kidneys (1.107±0.082 µg/equiv./g) and cardiac blood (0.921±0.078 µg/equiv./g). The concentrations found in the remaining tissues ranged from 0.042±0.011 µg/equiv./g (stomach and contents) to 0.624±0.428 µg/equiv./g (thyroids). The highest mean tissue concentrations of [14C]-fluopicolide equivalents in female tissues were found in cardiac blood (1.796±0.295 µg/equiv./g), followed by the liver (1.776±0.0.313 µg/equiv./g) the spleen was the only other organ to possess a mean concentration greater than 1 µg/equiv./g (1.014±0.250 µg/equiv./g). The concentrations found in the remaining tissues ranged from 0.087±0.010 µg/equiv./g (eyes) to 0.968±0.164 µg/equiv./g (adrenals).

[Phenyl-U-^{14}C]-fluopicolide was intensively metabolised with up to 57 radioactive components in the urine and up to 45 radioactive components in the faecal extracts.

Unchanged fluopicolide was identified as the main component in faecal samples from male and female rats, whilst no parent compound was detected in the urine.

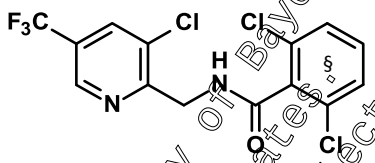
The observed routes of metabolism included glutathione conjugation and its subsequent biotransformation products, hydroxylation, conjugation with glucuronic acid, conjugation with sulphate, and oxidative N-dealkylation.

I. Materials and methods

A. Materials

1. Test material: [phenyl-U-^{14}C]-fluopicolide (referred to as AE C638206 in the report)

Chemical structure:



§ labelling position

Batch no.:	901CU2
Specific radioactivity:	2.202 GBq/mmole (59.5 mCi/mmole)
Radiochemical purity:	>98.3% by HPLC

2. Vehicle: Aqueous methyl cellulose

3. Test animals:

Species:	Rat *(Rattus norvegicus domesticus)*
Strain:	Sprague-Dawley CD
Age:	6 to 10 weeks
Weight at dosing:	156.28 - 207.29g
Source:	[redacted]
Acclimation period:	At least 5 days
Identification:	Animals were identified by ear tattoo and tail markings
Diet:	Certified rodent diet A04C, obtained from UAR, Villemoisson, France
Water:	Filtered and softened water obtained from the municipal supply that was routinely analysed, to ensure that no contaminants were present that could affect the outcome of the study
Housing:	Animals were kept individually in Jencon's Metabowls Mk III metabolism units
Environmental conditions:	
Temperature:	22 ± 2°C
Humidity:	55 ± 15%.
Ventilation:	15 air changes per hour
Photoperiod:	12 hour light & 12 hour dark.

4. Preparation of dosing solutions

For the 14-day dosing period, three dose preparations were made up using 0.75% (w/w) aqueous methyl cellulose. Dose preparation1 was formulated 24h before day 0 for administration on says 0-3, dose preparation 2 was formulated on day 3 for administration on days 4-8 and dose preparation 3 was formulated on day 8 for administration on days 9-13. The required amount of non-radiolabelled fluopicolide was weighed and a volume of radioactive solution (calculated to provide the required amount of specific radioactivity) was added. The solution was evaporated under a gentle stream of nitrogen and weight of vehicle (aqueous methyl cellulose) to provide a dose of 1g dose suspension/200 g rat body weight at the required dose level was added. The dose suspension was prepared 24-hours before the day of dosing and was assayed for active ingredient concentration (HPLC) and radioactivity content (LSC) before, mid-way through and following dosing.

B. Study design and methods

1. Dose regimen and design of tests

Group no.	No. doses	Time of sacrifice	No. rats	Nominal dose levels (mg/kg)	Nominal radioactive dose level (µCi/kg)	Investigations
A	14	144-hours after last administration	5 males 5 females	10	50	Urine, faeces, cage-wash, plasma, tissues, carcass

The rats were given fourteen daily doses of radiolabelled [phenyl-U-^{14}C]-fluopicolide.

[phenyl-U-^{14}C]-fluopicolide was administered orally to groups of five male of female rats at a low dose of 10 mg/kg bw each day. Urine was collected at 0-6h and 6-24h following the first dose and then every 24h thereafter. Faeces was collected at 24h intervals and, cage washes (washed with distilled water) were collected each 24-hours and at the end of the in-life phase after being washed with acetonitrile The animals were sacrificed 168-hours after the first dose, when blood, organs, GIT, skin, and carcass were also collected.

2. Dosing

Suspensions of the test compound in 0.75% aqueous methyl cellulose were administered orally to each rat. The test suspension was administered by gavage at a rate of 1g dose suspension per 2kg of rat body weight.

The dose suspension was assayed for test item concentration (by HPLC) and for radioactivity content (by LSC) at three time points (before, mid-way through and after each dosing procedure). Actual dose levels were close to nominal levels.

3. Collection of excreta

After the administration of the radiolabelled test substance, the rats were kept individually in metabowl units which allowed for separate and quantitative collection of urine and faeces.

Urine and faeces were collected separately for each rat over solid carbon dioxide contained in polystyrene vessels to protect the excreta from light.

Urine was collected at 0-6 hours and 6-24 hours and at twenty-four hour intervals following dosing.

Metabowl cages were washed with distilled water every 24 hours and with acetonitrile after the animals had been removed from the units at the end of the in-life phase. The radioactivity in urine and metabowl rinses was determined by LSC.

Faeces were collected separately for each rat every 24 hours following dosing. Faeces samples were homogenised with an appropriate volume of deionised water. The radioactivity was determined by combustion followed by LSC.

4. Expired air

The collection of expired carbon dioxide and other volatiles was not undertaken in this study as it had been established in in a previous study that this was not a likely route of administration (Fisher, P. J.; 2004; M-227025-02-1).

5. Sacrifice

The rats were anaesthetized by injection of Inalgene-500 and sacrificed by exsanguinated.

6. Blood, tissues, and organs at sacrifice

The following organs and tissues were collected: liver, kidneys, heart, lungs, brain, cardiac blood, spleen, pancreas, skeletal muscle, abdominal fat, ovaries (for females), testes (for males), stomach plus contents, intestine plus content, bone (femur) and marrow, adrenals, the skin & fur, uterus (for females), eyes, Harderian glands and thyroids. The residual carcass was also retained for analysis.

Scissor minced portions of fat, tests, bone (plus marrow) and uterus, as well as whole tissues such as ovaries, eyes, Harderian glands, thyroids, adrenals, and aliquots of cardiac blood were combusted directly on combusto-pads contained in combusto cones.

Other tissues (liver, kidney, heart, muscle, lung, brain, pancreas, stomach, and contents and intestine and contents) were homogenised using an Ultra-Turrax and combusted.

The skin, fur and the carcass were Solubised prior to radioassay.

7. Sample handling and storage

Whenever possible samples were processed as they were collected. Remaining samples and samples awaiting processing were stored at -20°C in the dark until required.

8. Preparation of samples for analysis

Two sets of pooled urine samples for the male and female groups for the periods 0-6 hours and days 1, 2, 3, 5, 8, 12 and 14 were prepared for metabolite quantification work. The samples were concentrated under a gentle stream of nitrogen and then centrifuged for 20 minutes. The supernatants were aliquoted and counted and the pellets extracted, and the recovery determined by LCS.

Samples were pooled according to sex and time period for metabolite quantification and identification. The pooled samples covered the time periods of days 1, 2, 3, 5, 8, 12 and 14. Faeces samples were extracted from the initial homogenisation of the samples prior to combustion analysis.

9. Analytical methods

The chromatographic separation and quantification of the metabolites present in urine and faecal samples was performed using the High Performance Liquid Chromatography (HPLC) with radio-detection and by LC-MS/MS methods.

II. Results and Discussion

A. Recovery

Male and female rats received repeated daily oral doses by gavage for a 14-day period with radiolabelled fluopicolide at a nominal dose rate of 10 mg/kg body weight. The mean recovery was found to be 95.51±1.19% in male rats and 96.30±0.39% in female rats. A summary of the radioactivity as percent of the administered dose found in urine, faeces and tissues is presented in the table below.

Table 5.1.1- 51: Recovery of radioactivity after repeated administration of [phenyl-U-^{14}C]-fluopicolide at rate of 10 mg/kg b/w

Timing	Sample	0 male oral, repeat 10 mg/kg bw/d		female oral, repeat 10 mg/kg bw/	
		Mean	**SD**	**Mean**	**SD**
0-480h	Urine	14.684	1.525	21.469	5.012
	Faeces	78.863	2.666	72.479	5.551
	Cage wash	1.662	0.408	1.892	0.477
	Sub-total	*95.209*	*1.201*	*85.841*	*0.368*
	Tissues	0.30	0.03	0.46	0.03
	Total	**95.51**	1.19	**96.30**	**0.39**

Percent of administered dose (mean values)

SD = standard deviation

The major route of excretion was *via* the faeces for both sexes with a mean of 78.863% of the administered dose in male rats and 72.479% in female rats eliminated during the study. The proportion of radioactivity eliminated *via* the urinary route (including cage wash) was 16.346% in male rats and 23.361% in female rats.

B. Absorption

The estimated minimum levels of absorption were measured as the total radioactivity in urine, cage washes plus tissues. The minimum amount of dose absorbed after multiple dosing was calculated to be *ca.* 16.64% in males and *ca.* 23.82% in females. Data from bile excretion studies indicate that the actual level of oral bioavailability will be much higher as biliary excretion was found to be a major route of elimination ([redacted] 2002; M-212243-01-1 phenyl; [redacted] 2020; M-681498-01-1 phenyl)

C. Distribution

Levels of radioactivity remaining in tissues at sacrifice represented a sum total of 0.30% of the administered dose in male rats and 0.46% in female rats. The distribution of radioactive residues in the rat are summarised in the table below, expressed as parent equivalent concentrations in µg/g.

The highest mean tissue concentrations of [14C]-fluopicolide equivalents in male tissues were found in the liver (1.356±0.082 µg/equiv./g), followed by the kidneys (1.107±0.082 µg/equiv./g) and cardiac blood (0.921±0.078 µg/equiv./g). The concentrations found in the remaining tissues ranged from 0.042±0.011 µg/equiv./g (stomach and contents) to 0.624±0.428 µg/equiv./g (thyroids).

The highest mean tissue concentrations of [14C]-fluopicolide equivalents in female tissues were found in cardiac blood (1.796±0.295 µg/equiv./g), followed by the liver (1.776±0.0.313 µg/equiv./g). the spleen was the only other organ to possess a mean concentration greater than 1 µg/equiv./g (1.014±0.250 µg/equiv./g). The concentrations found in the remaining tissues ranged from 0.087±0.010 µg/equiv./g (eyes) to 0.968±0.164 µg/equiv./g (adrenals).

Table 5.1.1- 52: Concentration of [phenyl-U-^{14}C]-fluopicolide residues in rat tissues following repeated oral dosing at 10 mg/kg bw/d

µg fluopicolide equivalents/g tissue

Organs/ Tissues	Oral Male 10 mg/kg bw/d		Oral female 10 mg/kg bw/d	
	Mean	SD	Mean	SD
Cardiac blood	0.904	0.842	2.044	1.349
Liver	1.367	1.233	2.130	1.548
Kidneys	1.002	1.170	1.997	1.851
Spleen	0.302	0.473	1.352	1.020
Adrenal	0.506	0.663	1.099	1.035
Heart	0.506	0.594	0.970	0.851
Harder's gland	0.412	0.532	0.790	0.903
Thyroids	0.349	0.461	0.694	0.866
Lungs	0.325	0.365	0.779	0.608
Skeletal muscle	0.242	0.295	0.587	0.585
Carcass	0.270	0.272	0.512	0.449
Pancreas	0.213	0.229	0.402	0.416
Ovaries	n.a	n.a	0.278	0.318
Abdominal fat	0.094	0.094	0.286	0.280
Testes	0.086	0.109	n.a	n.a
Skin and fur	0.158	0.200	0.207	0.235
Intestine and contents	0.134	0.277	0.189	0.182
Bone & marrow	0.100	0.110	0.165	0.151

Organs/ Tissues	Oral Male 10 mg/kg bw/d		Oral female 10 mg/kg bw/d	
Brain	0.092	0.098	0.170	0.145
Stomach & contents	0.022	0.045	0.090	0.074
Uterus	n.a	n.a	0.126	0.183
Cardiac plasma	0.040	0.089	0.098	0.142
Eyes	0.053	0.064	0.086	0.082
Total	0.30	0.03	0.46	0.03

SD = standard deviation, n.a = not applicable

D. Excretion

The routes and rates of elimination were similar between male and female rats. These data are presented in the table below.

In males, the major route of elimination is via the faeces; 14.684% of the dose was found in the urine and 78.863% in the faeces over the study period. The data show that the elimination rates plateaued by day 4 following administration of the first dose. The levels of radioactivity in urine and faeces declined rapidly upon cessation of dosing (ca. 97% reduction in elimination levels two days following last dose).

Similarly, faeces was the major route of elimination in females; 21.459 and 72.479% of the dose were found in urine and faeces respectively over the study period. As in males, elimination rates plateaued by day 3-4 following the first dosing, and the levels of radioactivity in urine and faeces rapidly declined after cessation of dosing (98% reduction within 2 days of the last dose).

Overall, therefore, there did not appear to be a sex difference with regard to the elimination profiles.

Table 5.1.1- 53: Elimination of radioactivity after repeated administration of [phenyl-U-^{14}C]-fluopicolide at rate of 10 mg/kg b/w

Percent of administered dose (mean values)

Sample	Time [hours]	male oral 10 mg/kg bw		female oral 10 mg/kg bw	
		Mean	SD	Mean	SD
Urine	0-6	0.083	0.040	0.132	0.019
	6-24	0.238	0.074	0.656	0.227
	24-48	0.774	0.089	1.349	0.215
	48-72	0.854	0.132	1.495	0.201
	72-96	0.999	0.102	1.597	0.452
	96-120	0.94	0.231	1.579	0.427
	120-144	1.130	0.220	1.526	0.329
	144-168	1.154	0.131	1.651	0.461
	168-192	1.282	0.340	1.626	0.449
	192-216	1.049	0.203	1.702	0.475
	216-240	1.043	0.074	1.360	0.355
	240-264	1.016	0.109	1.418	0.405
	264-288	1.030	0.132	1.471	0.370
	288-312	1.111	0.143	1.529	0.424

Sample	Time [hours]	male oral 10 mg/kg bw		female oral 10 mg/kg bw	
		Mean	SD	Mean	SD
	312-336	1.130	0.155	1.617	0.469
	336-360	0.463	0.102	0.492	0.137
	360-384	0.171	0.042	0.120	0.028
	384-408	0.072	0.012	0.056	0.014
	408-432	0.048	0.012	0.040	0.009
	432-456	0.027	0.006	0.030	0.004
	456-480	0.016	0.006	0.023	0.010
	Sub total	14.684	1.525	21.469	5.012
Faeces	0.24	2.861	0.732	3.419	0.515
	24-48	5.096	0.427	4.565	0.484
	48-72	5.184	0.390	4.848	1.442
	72-96	5.567	0.538	4.785	0.617
	96-120	5.093	0.236	4.854	0.517
	120-144	6.041	0.333	5.149	1.021
	144-168	6.054	0.371	5.936	0.884
	168-192	5.687	0.503	5.388	0.443
	192-216	6.176	0.512	5.778	0.665
	216-240	0.344	0.298	5.755	0.765
	240-264	5.723	0.684	5.259	0.588
	264-288	5.599	0.546	4.922	0.818
	288-312	5.750	0.510	5.011	0.640
	312-336	5.721	0.420	5.209	1.008
	336-360	1.419	0.414	1.105	0.248
	360-384	0.300	0.126	0.110	0.044
	384-408	0.146	0.040	0.043	0.022
	408-432	0.056	0.018	0.020	0.03
	432-456	0.029	0.009	0.016	0.003
	456-480	0.019	0.008	0.015	0.003
	Sub total	78.863	2.666	72.479	5.551
Cage wash	Total	1.662	0.408	1.892	0.477
Total eliminated	Total	95.209	1.201	95.841	0.368

SD = standard deviation

E. Metabolism

The unchanged test item, fluopicolide (AE C638206), was the major component in faeces; no parent compound was detected in the urine.

Investigations into the metabolism of [Phenyl-U-^{14}C]-AE C638206 in urine and faecal extract samples revealed the presence of up to 57 radioactive components in the urine and up to 45 radioactive components in the faecal extracts. Thus, it appeared that the [Phenyl-U-^{14}C]-fluopicolide was extensively metabolised in the rat following repeated oral dosing.

The urinary metabolite profile was qualitatively and quantitatively similar between the sexes given that over 96% of the radioactivity found in the urine samples was common to both sexes albeit that some individual metabolites varied in quantity between the sexes.

In total 87.5% of the radioactivity eliminated in the urine by the male rats over the 0-336 hour period post the first dose was assigned a molecular weight which accounted for 11.16% of the total dose. Structures were proposed for 73.15% of the radioactivity eliminated in the 14 days following the first dose, which accounted for 9.33% of the total dose.

For the females, a total of 90.07% of the radioactivity eliminated in the urine over the 0-336 hour period post the first dose was assigned a molecular weight which accounted for 18.65% of the total dose. Structures were proposed for 84.19% of the radioactivity eliminated in the 14 days following the first dose, which accounted for 17.43% of the total dose.

The faecal metabolite profile was qualitatively and quantitatively similar between the sexes given that over 97% of the radioactivity found in the faecal samples was common to both sexes albeit that some individual metabolites varied in quantity between the sexes.

The large number of metabolites demonstrated that the rat was capable of extensively metabolising fluopicolide although *ca* 34% (males) to *ca* 40% (females) of the [phenyl-U-^{14}C]-fluopicolide was eliminated unchanged over first 14 days of the experiment.

In total 87.1% of the radioactivity eliminated in the faeces by the male rats over the 0-336 hour period post the first dose was assigned a molecular weight which accounted for 66.96% of the total dose. Structures were proposed for 85% of the radioactivity eliminated in the 14 days following the first dose, which accounted for 65.35% of the total dose.

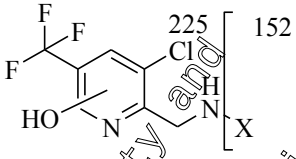
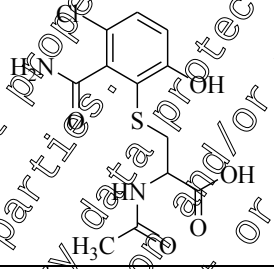
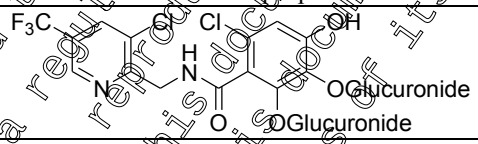
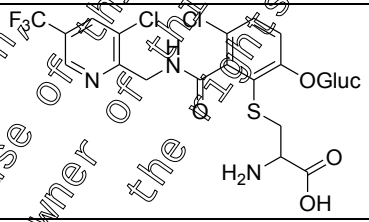
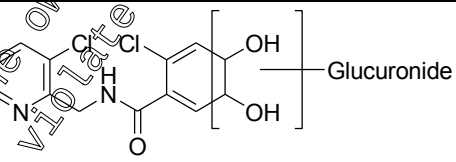
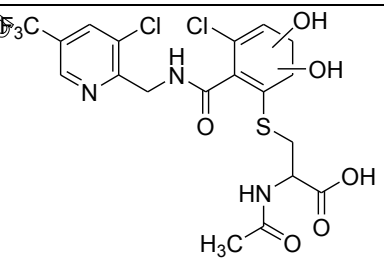
For the females, a total of 88.2% of the radioactivity eliminated in the urine over the 0-336 hour period post the first dose was assigned a molecular weight, which accounted for 62.74% of the total dose. Structures were proposed for 87.1% of the radioactivity eliminated in the 14 days following the first dose, which accounted for 61.98% of the total dose.

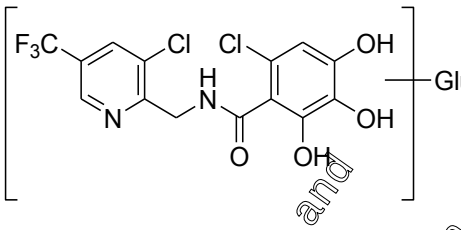
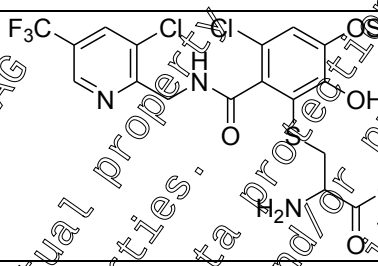
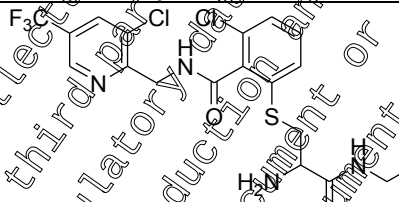
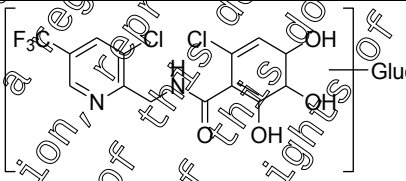
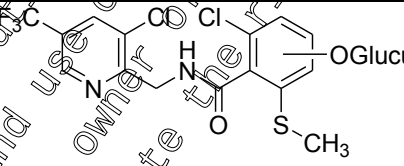
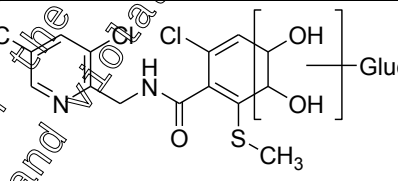
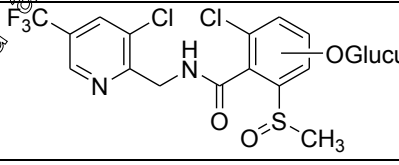
By summing the components that were assigned structures in the urine and the faeces, a total of 74.7% of the administered radioactivity has been assigned a structure for the males and 79.4% of the administered radioactivity for the females.

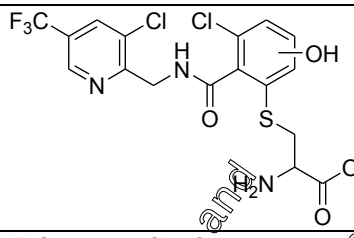
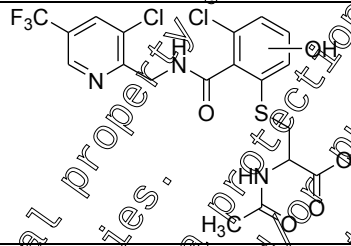
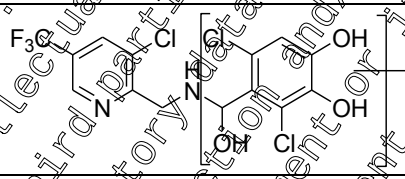
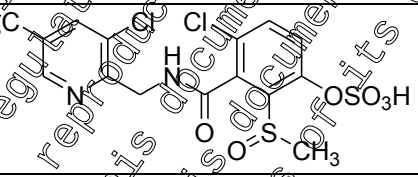
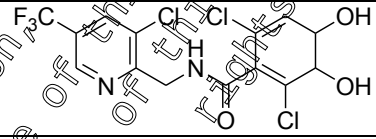
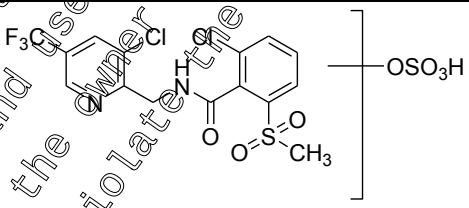
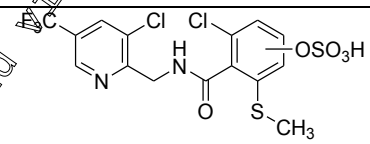
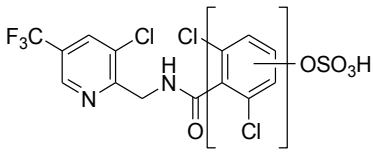
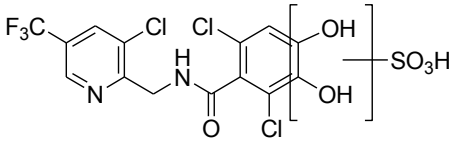
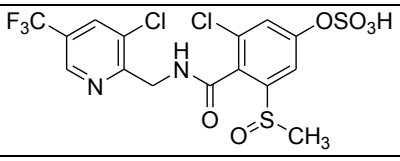
Comparison of the routes of metabolism between the single and repeated dose study demonstrated that repeated dosing did not have any significant effect upon the biotransformation of AE C638206.

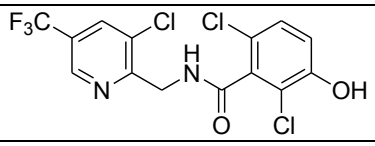
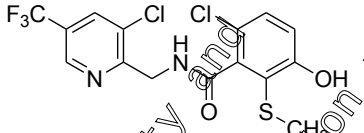
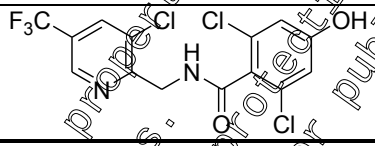
The identity and quantification of the metabolites are presented in the tables below.

Table 5.1.1- 54: Identified metabolites in urine of male and female rats

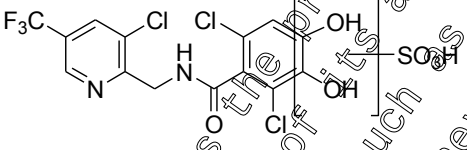
UMET/-	%dose		Assigned Molecular Mass	Proposed Structure
	Males	Females		
1	0.55	0.33	377 or 395 Cl	
4	0.16	0.43	264 (Cl)	No structure proposed
7	2.34	0.40	332	
10	0.69	0.15	601 ($Cl_{2 or 3}$)	No structure proposed
17	0.24	0.16	317 (Cl_2)	No structure proposed
18	0.67	0.34	387(Cl_2)	No structure proposed
20	0.35	0.30	748	
26	0.29	0.31	659	
28	0.03	0.26	558	
29	0.13	0.30	543	
31	0.08	0.14	578 (Cl)	No structure proposed

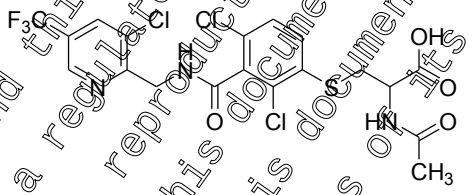
32	0.23	1.39	572	
			563	
34	0.49	1.52	540	
			574	
			586	
			588	
			602	

36	0.09	1.58	483	
			525	
40	1.37	1.78	496	
			506	
42	0.53	1.93	416	
44	0.49	0.22	522	
46	1.38	6.55	490	
47	0.02	0.04	478	
49	0.51	0.59	494	
			506	

53	0.44	0.43	398 AE C643890	
54	0.08	0.30	410	
			398	
Total	11.16	18.65		

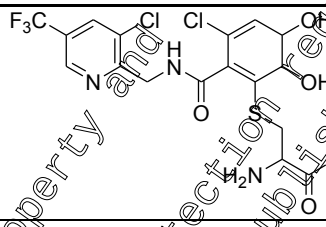
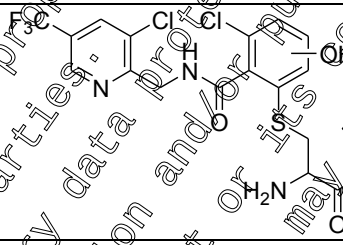
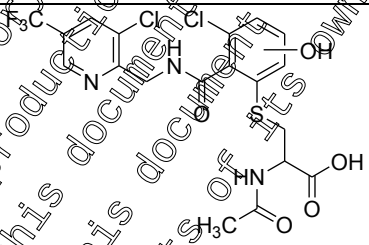
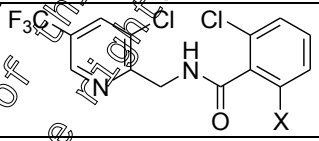
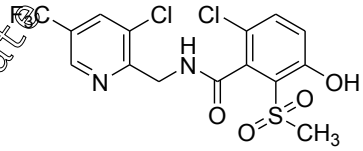
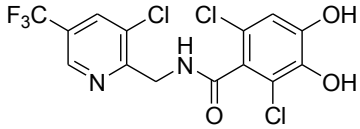
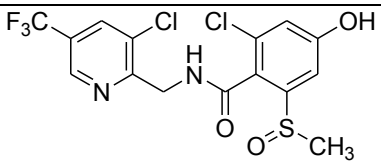
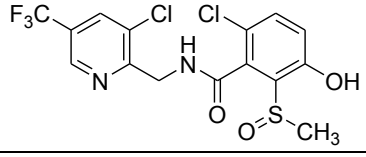
Additionally, there were two metabolite structures observed during the mass spectroscopic analyses that had not been observed/quantified by the radio-HPLC investigations:





An isomer of UMET/49 with an earlier retention time.

Table 5.1.1- 55: Identified metabolites in faeces of male and female rats

FMET/- N°	%dose		Molar Mass	Proposed Structure
	Males	Females		
9	0.56	0.39	501	
14	6.88	2.88	485	
16	1.71	1.08	525	
			513 X = ?	
17	1.61	0.76	547 (Cl_2)	No structure proposed
22	0.97	n.d.	442 AE 916598	
28	2.71	2.23	414	
			426	
			426 AE 0717559	

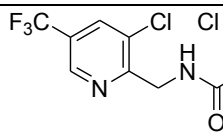
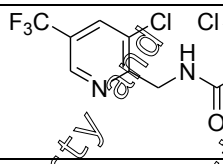
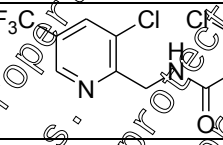
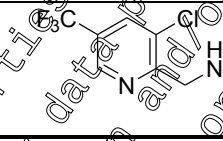
33	2.26	1.93	398 AE C643890	
35	11.52	7.89	410 AE 0717560	
37	5.11	6.10	394	
39	33.62	39.45	382 AE C638206	
Total	66.96	62.74		

Table 5.1.1- 56: Quantification of metabolites in urine of male rats

UMET/-N°	MALES (% of Total Dose Administered)								
	0-6 h	6-24 h	Day 2	Day 3	Day 5	Day 8	Day 12	Day 14	Sum
1	0.0022	0.0145	0.0375	0.0366	0.0465	0.0509	0.0458	0.0419	**0.276**
2	0.0046	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.005
3	0.0021	0.0044	n.d.	0.0033	0.0037	n.d.	n.d.	n.d.	0.014
4	n.d.	0.0097	0.0365	n.d.	n.d.	0.0023	0.0106	n.d.	0.059
5	0.0048	n.d.	n.d.	0.0344	0.0353	0.0521	0.0303	0.0358	**0.193**
6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
7	0.0216	0.0672	0.1515	0.1466	0.1571	0.1961	0.1415	0.1270	**1.009**
8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
10	0.0086	n.d.	0.0397	0.0479	0.0479	0.0674	0.0650	0.0655	**0.342**
11	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
12	n.d.	0.0003	n.d.	n.d.	n.d.	0.0042	n.d.	n.d.	0.004
13	0.0003	0.0027	0.0063	0.0068	0.0064	0.0096	0.0087	0.0059	0.047
14	0.0007	0.0005	0.0062	0.0053	0.0065	0.0065	0.0038	0.0028	0.032
15	n.d.	n.d.	0.0039	0.0041	0.0043	0.0040	0.0043	0.0050	0.026
16	0.0003	0.0010	0.0031	0.0022	0.0028	0.0026	0.0009	0.0019	0.015
17	n.d.	0.0050	0.0150	0.0187	0.0221	0.0283	0.0236	0.0218	**0.135**
18	0.0033	0.0089	0.0299	0.0358	0.0484	0.0821	0.0773	0.0834	**0.369**
19	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
20	0.0013	0.0099	0.0235	0.0228	0.0249	0.0309	0.0292	0.0308	**0.173**
21	n.d.	0.0008	n.d.	0.0013	n.d.	0.0012	n.d.	n.d.	0.003
22	0.0003	0.0038	0.0092	0.0123	0.0075	n.d.	n.d.	n.d.	0.033
23	0.0016	0.0018	n.d.	n.d.	0.0017	0.0204	0.0099	0.0052	0.041
24	0.0024	0.0015	0.0066	0.0034	0.0074	n.d.	0.0062	0.0111	0.039
25	0.0004	0.0016	0.0041	n.d.	0.0006	n.d.	0.0044	0.0023	0.013
26	0.0031	0.0075	0.0244	0.0169	0.0158	0.0267	0.0139	0.0132	**0.121**
27	0.0013	0.0025	0.0052	0.0097	0.0121	0.0091	0.0112	0.0154	0.067
28	0.0003	0.0016	0.0017	0.0018	0.0021	n.d.	n.d.	n.d.	0.007
29	0.0010	0.0028	0.0105	0.0085	0.0096	0.0090	0.0100	0.0088	0.060
30	n.d.	0.0015	0.0044	n.d.	n.d.	n.d.	n.d.	n.d.	0.006
31	0.0003	0.0007	0.0128	0.0014	0.0049	0.0055	0.0058	0.0092	0.041

UMET/-N°	MALES (% of Total Dose Administered)								
	0-6 h	6-24 h	Day 2	Day 3	Day 5	Day 8	Day 12	Day 14	Sum
32	0.0014	0.0048	0.0132	0.0128	0.0161	0.0222	0.0199	0.0264	**0.117**
33	n.d.	0.0029	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.003
34	0.0039	0.0097	0.0091	0.0133	0.0418	0.0508	0.0531	0.0679	**0.250**
35	n.d.	n.d.	0.0043	n.d.	n.d.	n.d.	n.d.	n.d.	0.004
36	0.0007	0.0100	n.d.	0.0053	n.d.	n.d.	n.d.	0.0012	0.017
37	n.d.	n.d.	0.0050	n.d.	0.0016	n.d.	n.d.	0.0011	0.008
38	0.0002	n.d.	n.d.	0.0012	n.d.	n.d.	0.0018	n.d.	0.003
39	n.d.	0.0008	n.d.	n.d.	n.d.	0.0008	n.d.	0.0023	0.004
40	0.0043	0.0148	0.0868	0.1118	0.1320	0.1777	0.1359	0.1601	**0.820**
41	n.d.	0.0008	n.d.	0.0010	n.d.	n.d.	n.d.	n.d.	0.002
42	0.0050	0.0101	0.0320	0.0245	0.0244	0.0287	0.0213	0.0240	**0.176**
43	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
44	n.d.	0.0042	0.0214	0.0371	0.0468	0.0658	0.0593	0.0732	**0.308**
45	0.0006	n.d.	n.d.	n.d.	0.0012	n.d.	n.d.	n.d.	0.001
46	0.0003	0.0139	0.0829	0.1008	0.1417	0.1890	0.1354	0.1707	**0.835**
47	0.0017	0.0006	n.d.	0.0013	0.0013	n.d.	n.d.	0.0037	0.007
48	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
49	n.d.	n.d.	0.0266	0.0395	0.0478	0.0561	0.0511	0.0609	**0.289**
50	n.d.	0.0053	0.0023	0.0188	0.0171	0.0147	0.0102	n.d.	0.063
51	n.d.	0.0013	0.0143	0.0094	0.0033	0.0054	0.0023	0.0096	0.046
52	n.d.	0.0003	n.d.	0.0032	0.0033	0.0064	0.0018	0.0031	0.020
53	0.0017	0.0060	0.0373	0.0431	0.0405	0.0479	0.0286	0.0299	**0.235**
54	n.d.	0.0013	0.0065	0.0091	0.0075	0.0079	0.0068	0.0082	0.047
55	n.d.	0.0002	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.0002
56	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
57	n.d.	0.0010	n.d.	n.d.	n.d.	n.d.	n.d.	0.0011	0.002
Total	0.0830	0.2380	0.7739	0.8539	0.9939	1.2822	1.0298	1.1301	6.385

Table 5.1.1- 57: Quantification of metabolites in urine from female rats

UMET/-N°	FEMALES (% of Total Dose Administered)								
	0-6 h	6-24 h	Day 2	Day 3	Day 5	Day 8	Day 12	Day 14	Sum
1	0.0039	0.0072	0.0190	0.0187	0.0219	0.0232	0.0225	0.0239	**0.140**
2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
3	0.0023	0.0026	0.0067	0.0038	0.0051	0.0077	0.0035	0.0061	0.038
4	0.0048	0.0123	0.0296	0.0338	0.0376	0.0218	0.0063	0.0408	**0.187**
5	n.d.	n.d.	0.0115	n.d.	n.d.	n.d.	n.d.	n.d.	0.012
6	0.0072	n.d.	0.0560	0.0471	0.0636	0.0526	0.0326	n.d.	**0.259**
7	n.d.	0.0222	n.d.	n.d.	0.0161	0.0479	0.0604	0.0665	**0.213**
8	n.d.	n.d.	n.d.	0.0098	n.d.	n.d.	n.d.	n.d.	0.010
9	n.d.	n.d.	n.d.	0.0044	n.d.	n.d.	n.d.	0.0107	0.015
10	0.0050	n.d.	0.0132	0.0040	0.0116	n.d.	n.d.	n.d.	0.034
11	n.d.	0.0060	n.d.	0.0014	n.d.	0.0200	0.0252	0.0237	0.076
12	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
13	n.d.	n.d.	0.0015	n.d.	n.d.	0.0024	0.0031	0.0017	0.009
14	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.0015	0.0006	0.002
15	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.0021	0.0015	0.004
16	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
17	0.0004	0.0024	0.0098	0.0117	0.0148	0.0164	0.0156	0.0179	0.089
18	0.0036	0.0047	0.0152	0.0184	0.0224	0.0279	0.0274	0.0355	**0.155**
19	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.0034	n.d.	0.003
20	0.0005	0.0118	0.0231	0.0202	0.0287	0.0240	0.0220	0.0286	**0.159**

UMET/- N°	FEMALES (% of Total Dose Administered)								
	0-6 h	6-24 h	Day 2	Day 3	Day 5	Day 8	Day 12	Day 14	Sum
21	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
22	0.0019	n.d.	0.0036	0.0037	0.0081	0.0001	n.d.	n.d.	0.017
23	0.0014	n.d.	0.0038	0.0032	n.d.	0.0023	0.0025	n.d.	0.013
24	0.0024	0.0027	0.0097	0.0088	0.0084	0.0046	0.0073	0.0032	0.047
25	0.0004	n.d.	0.0032	0.0018	0.0032	0.0007	n.d.	0.0084	0.018
26	0.0043	0.0091	0.0182	0.0182	0.0167	0.0087	0.0262	0.0228	**0.124**
27	0.0025	0.0063	0.0157	0.0175	0.0185	0.0225	n.d.	0.0084	0.091
28	0.0034	0.0075	0.0155	0.0176	0.0173	0.0121	0.0140	0.0175	**0.105**
29	0.0034	0.0095	0.0193	0.0215	0.0205	0.0170	0.0173	0.0171	**0.126**
30	0.0012	n.d.	0.0025	0.0047	0.0055	0.0042	0.0015	0.0033	0.020
31	0.0015	0.0053	0.0124	0.0130	0.0181	0.0075	n.d.	0.0024	0.060
32	0.0096	0.0484	0.0859	0.0925	0.1014	0.1135	0.1027	0.0994	**0.653**
33	n.d.	0.0156	0.0178	n.d.	n.d.	0.0122	0.0128	0.0057	0.064
34	0.0128	0.0600	0.0853	0.1399	0.1430	0.1323	0.0722	0.0361	**0.682**
35	0.0030	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.003
36	0.0180	0.0745	0.1005	0.1070	0.0992	0.0811	0.0776	0.0811	**0.639**
37	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
38	n.d.	0.0010	n.d.	n.d.	n.d.	n.d.	n.d.	0.0011	0.002
39	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
40	0.0040	0.0653	0.1296	0.1403	0.1423	0.1476	0.1292	0.1580	**0.916**
41	0.0017	0.0030	0.0080	0.0025	0.0068	0.0026	0.0031	0.0082	0.033
42	0.0221	0.0202	0.0546	0.0547	0.0561	0.0619	0.0642	0.0709	**0.405**
43	n.d.	0.0014	n.d.	0.0045	0.0069	0.0018	0.0038	0.0050	0.023
44	0.0010	0.0067	0.0168	0.0167	0.0193	0.0109	0.0154	0.0209	**0.108**
45	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
46	0.0036	0.2273	0.4563	0.5463	0.5451	0.6163	0.5354	0.5883	**3.519**
47	0.0021	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.002
48	0.0001	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.000
49	0.0023	0.0158	0.0405	0.0454	0.0516	0.0496	0.0467	0.0536	**0.306**
50	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
51	n.d.	n.d.	0.0105	0.0137	0.0087	0.0055	0.0189	0.0353	0.092
52	n.d.	n.d.	0.0032	0.0013	0.0060	0.0069	0.0117	0.0150	0.044
53	0.0014	0.0036	0.0275	0.0239	0.0319	0.0393	0.0524	0.0553	**0.235**
54	n.d.	0.0073	0.0262	0.0237	0.0225	0.0241	0.0307	0.0390	**0.169**
55	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.0023	0.002
56	0.0004	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.0049	0.005
57	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total	0.1320	0.6560	1.3491	1.4952	1.5788	1.6258	1.4711	1.6202	9.928

Table 5.1.1- 58: Quantification of metabolites in faeces from male rats

FMET/- N°	MALES (% of Total Dose Administered)							
	Day 1	Day 2	Day 3	Day 5	Day 8	Day 12	Day 14	Sum
1	n.d.	0.0148	n.d.	n.d.	n.d.	n.d.	n.d.	0.015
2	0.0154	0.0064	0.0200	0.0306	0.0535	0.0361	0.0635	0.225
3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.0097	0.010
4	0.0064	n.d.	n.d.	n.d.	n.d.	n.d.	0.0257	0.032
5	0.0072	0.0074	n.d.	n.d.	n.d.	n.d.	n.d.	0.015
6	0.0041	n.d.	n.d.	n.d.	n.d.	n.d.	0.0332	0.037
7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
8	0.0049	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.005
9	0.0180	0.0359	0.0272	0.0344	0.0620	0.0137	0.0687	0.260
10	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.0229	0.023
11	n.d.	n.d.	n.d.	n.d.	0.0162	n.d.	n.d.	0.016
12	0.0299	0.0296	n.d.	n.d.	n.d.	n.d.	n.d.	0.059
13	0.0033	0.0056	n.d.	n.d.	0.0119	n.d.	0.0306	0.051
14	0.5981	0.7738	0.3188	0.2867	0.2872	0.2279	0.3204	**2.813**
15	n.d.	n.d.	0.0254	n.d.	n.d.	n.d.	n.d.	0.025
16	0.0688	0.1164	0.1651	0.1128	0.1635	0.0921	0.0561	**0.775**
17	0.0667	0.1073	0.1048	0.1497	0.1351	0.0703	0.0924	**0.726**
18	0.0592	0.0703	0.0407	0.0278	0.0401	0.0280	0.0366	0.303
19	n.d.	n.d.	0.0726	n.d.	n.d.	n.d.	0.0626	0.135
20	0.0664	0.0961	0.0591	0.0949	0.1311	n.d.	0.0823	0.477
21	0.0162	0.0270	0.0360	0.0211	n.d.	n.d.	n.d.	0.100
22	0.0080	0.0173	0.0088	0.0150	0.2101	n.d.	0.2320	0.491
23	n.d.	0.0003	n.d.	n.d.	0.0267	n.d.	n.d.	0.027
24	n.d.	0.0257	0.0674	0.1143	n.d.	0.1470	n.d.	0.354
25	0.0140	0.0148	0.0467	0.0385	n.d.	0.0168	0.0215	0.152
26	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
27	n.d.	n.d.	n.d.	0.1164	0.1797	0.0126	0.1559	0.465
28	0.0285	0.1437	0.1968	0.2131	0.1777	0.2942	0.2669	**1.321**
29	n.d.	n.d.	0.0391	n.d.	0.0299	n.d.	n.d.	0.069
30	n.d.	n.d.	n.d.	n.d.	n.d.	0.0157	n.d.	0.016
31	0.0152	0.0668	0.0573	0.0759	0.0546	0.0538	0.0981	0.422
32	n.d.	n.d.	0.0319	n.d.	n.d.	n.d.	n.d.	0.032
33	0.0473	0.1455	0.1257	0.1663	0.2801	0.1520	0.1559	**1.073**
34	n.d.	n.d.	0.0236	n.d.	n.d.	0.0154	n.d.	0.039
35	0.1247	0.7430	1.0552	0.9541	0.9435	0.8163	0.9174	**5.534**
36	0.0117	0.0066	n.d.	0.0244	n.d.	n.d.	n.d.	0.043
37	0.1970	0.3952	0.4469	0.2865	0.3864	0.3099	0.3024	**2.324**
38	0.0132	0.0257	0.0555	0.0183	0.0330	0.0171	0.0609	0.224
39	1.4136	2.0603	1.9689	2.1314	2.2319	3.0800	2.4114	**15.298**
40	0.0142	0.0854	0.1140	0.1235	0.1004	0.1229	0.1056	**0.666**
41	n.d.	n.d.	n.d.	n.d.	0.0498	0.0291	n.d.	0.079
42	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
43	0.0090	0.0573	0.0280	0.0400	0.0637	0.0227	0.0526	0.273
44	n.d.	0.0183	0.0492	0.0407	0.0188	0.0258	0.0858	0.239
45	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total	2.8610	5.0965	5.1840	5.0935	5.6867	5.5993	5.7210	35.2420

Table 5.1.1- 59: Quantification of metabolites in faeces from female rats

FMET/- N°	FEMALES (% of Total Dose Administered)							
	Day 1	Day 2	Day 3	Day 5	Day 8	Day 12	Day 14	Sum
1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2	0.0106	0.0057	n.d.	0.0274	0.0256	0.0239	0.0474	0.141
3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
6	0.0044	n.d.	n.d.	n.d.	0.0129	n.d.	n.d.	0.017
7	n.d.	n.d.	n.d.	n.d.	0.0121	n.d.	0.0154	0.027
8	n.d.	n.d.	n.d.	n.d.	0.1142	n.d.	0.0922	0.206
9	0.0084	0.0314	0.0461	0.0362	n.d.	0.0529	0.0081	0.183
10	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.0128	0.013
11	n.d.	n.d.	n.d.	n.d.	0.0121	n.d.	0.0094	0.021
12	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
13	n.d.	0.0048	0.0136	n.d.	n.d.	n.d.	0.0836	0.202
14	0.2308	0.1647	0.3304	0.0950	0.2476	0.1974	0.0320	**1.298**
15	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
16	0.0494	0.0578	0.0802	0.0299	0.1325	0.0578	0.1065	**0.514**
17	0.0468	0.1102	n.d.	n.d.	0.0423	0.0979	0.0482	0.345
18	0.0603	0.0588	0.0938	0.0748	0.0913	0.0187	0.0164	0.414
19	n.d.	n.d.	0.0499	0.0238	n.d.	n.d.	0.0227	0.096
20	0.0412	0.0824	0.0892	0.0279	0.1134	0.0758	0.0649	0.495
21	n.d.	n.d.	0.0115	n.d.	n.d.	0.0172	n.d.	0.059
22	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
23	n.d.	0.0041	n.d.	n.d.	n.d.	n.d.	n.d.	0.004
24	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
25	n.d.	0.0210	0.0206	n.d.	n.d.	n.d.	n.d.	0.042
26	n.d.	n.d.	n.d.	n.d.	n.d.	0.0221	0.0388	0.061
27	n.d.	0.0251	n.d.	n.d.	n.d.	n.d.	n.d.	0.025
28	0.0099	0.1285	0.1593	0.1732	0.2505	0.1688	0.2052	**1.095**
29	n.d.	n.d.	n.d.	n.d.	0.0348	0.0199	0.0234	0.078
30	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.0156	0.016
31	0.0120	0.0469	0.0708	0.0668	0.0768	0.0807	0.0750	0.429
32	n.d.	0.0027	n.d.	n.d.	n.d.	n.d.	n.d.	0.003
33	0.0691	0.1460	0.1474	0.1421	0.1716	0.1233	0.1110	**0.910**
34	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
35	0.2347	0.5202	0.6135	0.5933	0.7182	0.5370	0.5334	**3.750**
36	n.d.	0.0121	0.0235	0.0138	n.d.	0.0231	n.d.	0.073
37	0.2826	0.3865	0.4807	0.6363	0.3699	0.2200	0.4652	**2.841**
38	0.0284	0.0344	n.d.	n.d.	n.d.	0.0165	0.0195	0.099
39	2.2658	2.4258	2.2449	2.6471	2.7490	3.0772	2.8215	**18.231**
40	0.0157	0.0859	0.0210	0.1474	0.0924	0.0669	0.0896	**0.618**
41	0.0376	n.d.	0.0242	n.d.	n.d.	n.d.	n.d.	0.062
42	n.d.	0.1050	0.1054	0.0661	0.0762	0.0244	0.0716	0.449
43	0.0116	0.0622	n.d.	n.d.	n.d.	n.d.	0.0557	0.130
44	n.d.	0.0342	0.0545	0.0559	0.0442	n.d.	0.0242	0.213
45	n.d.	n.d.	0.0378	n.d.	n.d.	n.d.	n.d.	0.038
Total	3.419	4.555	4.848	4.857	5.388	4.922	5.209	33.198

The proposed metabolic pathway is outlined below:

Figure 5.1.1-11: Proposed Metabolic Pathway for [^{14}C]-fluopicolide following repeated oral dosing at the rate of 10 mg/kg bw/d

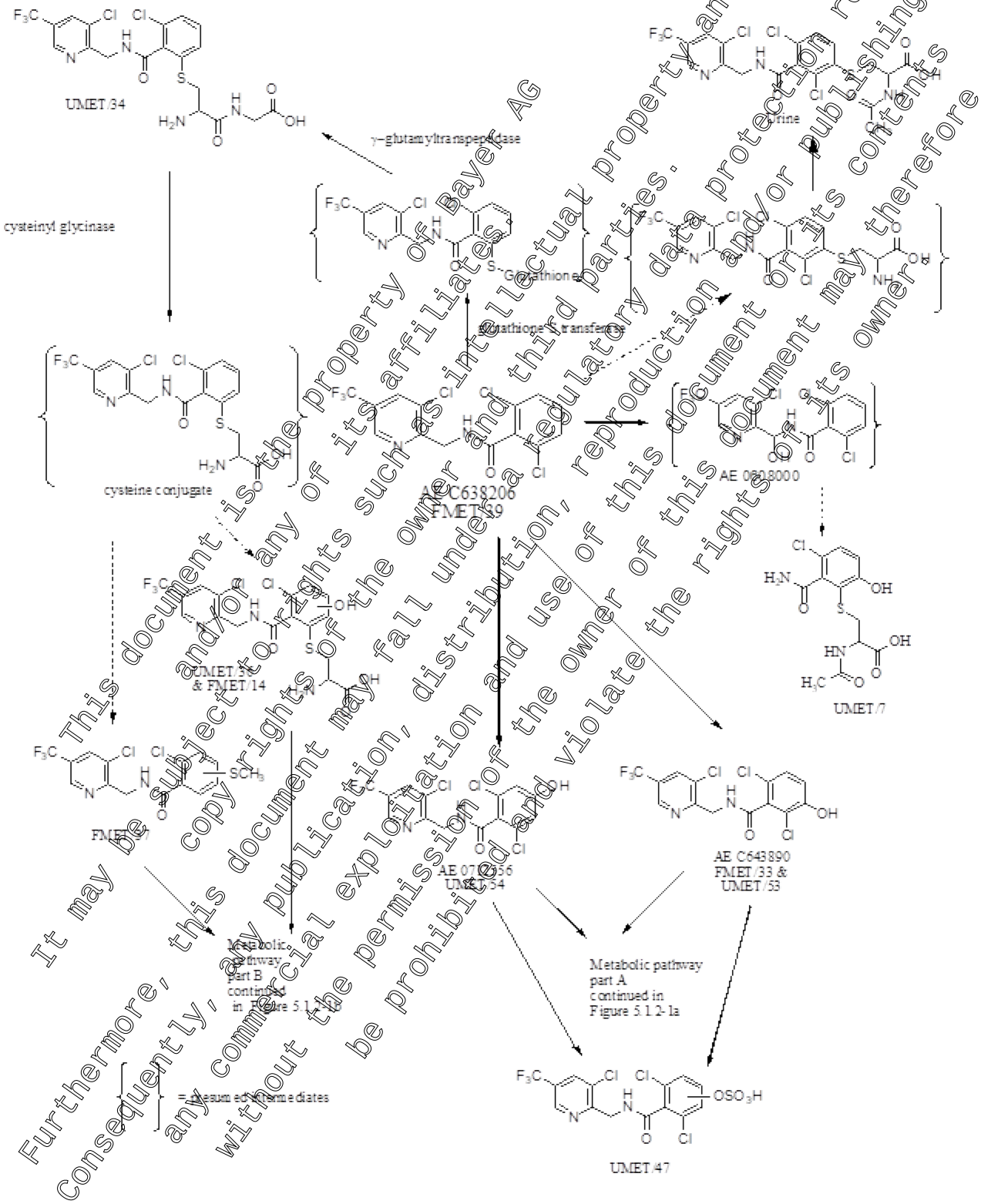


Figure 5.1.1-12: **Proposed Metabolic Pathway for [^{14}C]-fluopicolide following repeated oral dosing at the rate of 10 mg/kg bw/d cont.**

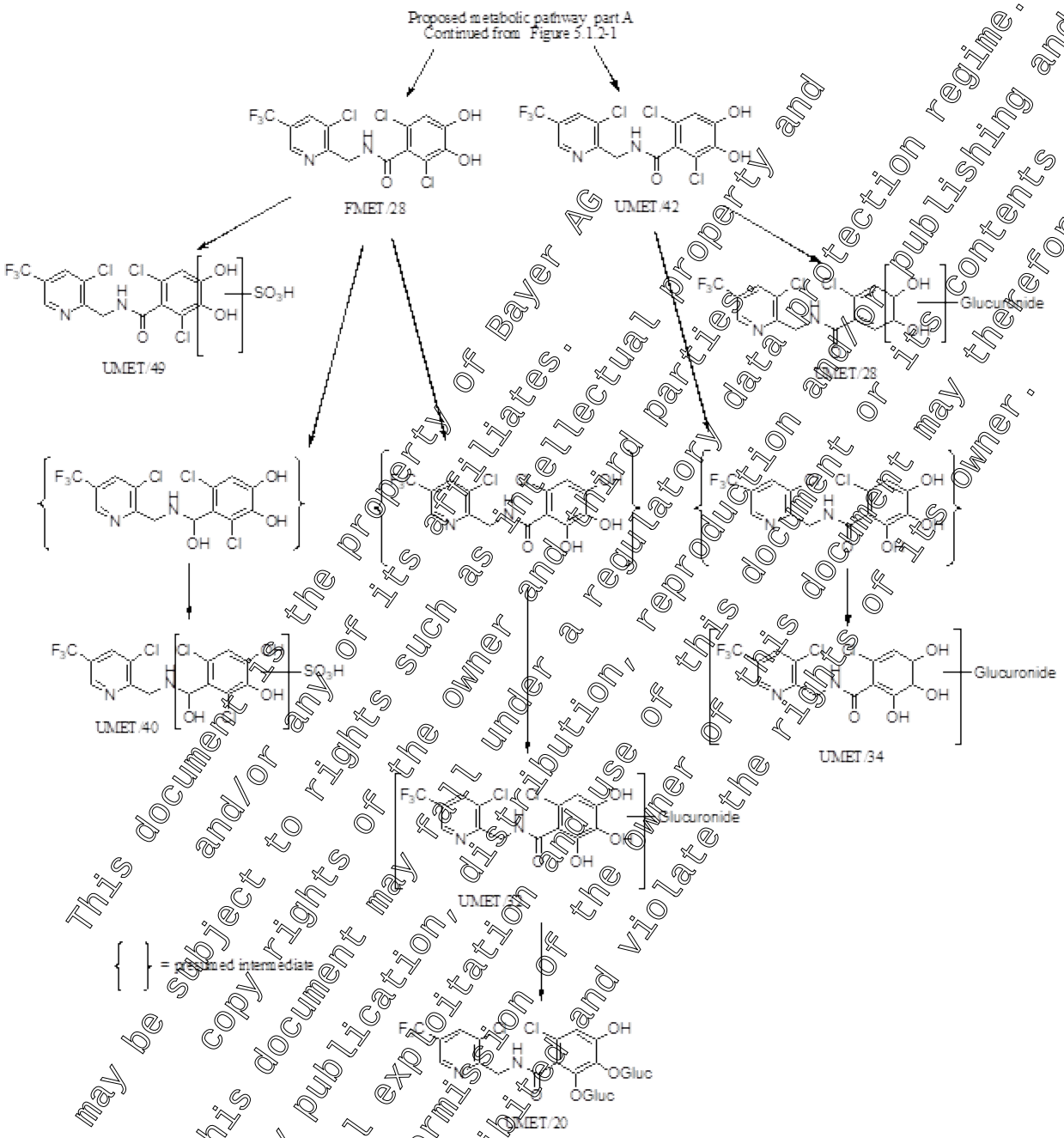
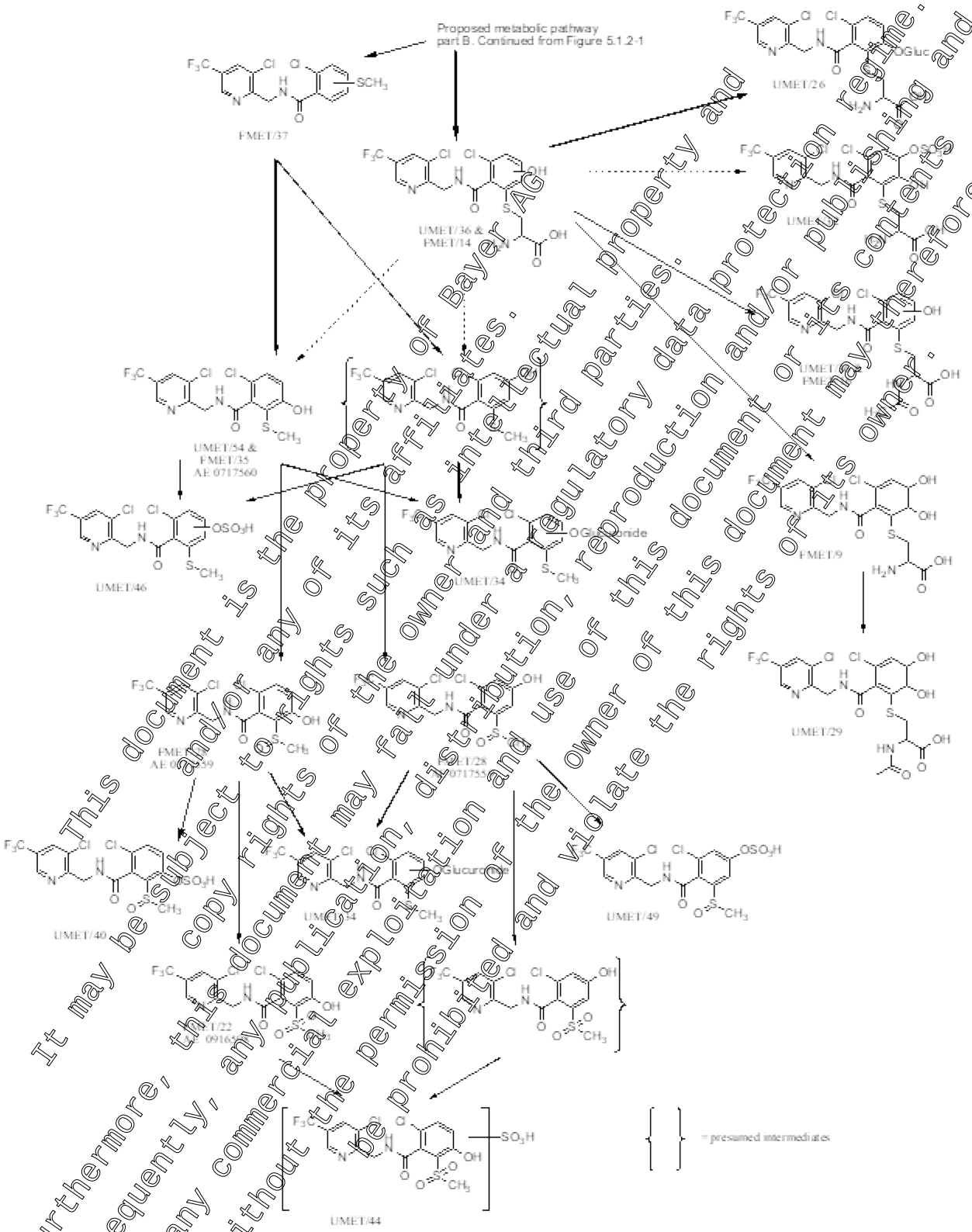


Figure 5.1.1-13: Proposed Metabolic Pathway for [^{14}C]-fluopicolide following repeated oral dosing at the rate of 10 mg/kg bw/d cont



III. Conclusions

Overall, multiple dosing of fluopicolide did not have any significant impact in the absorption, distribution, metabolism, and elimination compared to results after single oral dosing. The routes and rates of excretion were maintained with multiple dosing, with most of the radioactivity eliminated *via* the faeces within 48 hours post multiple dosing. The faeces accounted for *ca* 79% dose for the males and *ca* 73% dose for the females. The urine accounted for *ca* 15% dose for the males and *ca* 28% dose for the females. The majority of the elimination occurred in the 48 hours following cessation of dosing. The tissues contained only low levels of radioactivity 6 days following the last dose occasion accounting for <0.5% dose for both sexes. The liver, kidneys (organs of excretion and metabolism) and blood contained the highest concentrations of radioactivity in both sexes. A large number of metabolites were observed in the excreta (up to 57 in the urine and 45 in the faeces) and the routes of biotransformation were found to be qualitatively similar between the sexes. The observed routes of metabolism included glutathione conjugation and its subsequent biotransformation products, hydroxylation, conjugation with glucuronic acid, conjugation with sulphate and oxidative N-dealkylation.

Assessment and conclusion by applicant:
An acceptable study yielding valid conclusions

Data Point:	KCA 5.1.1/13
Report Author:	[redacted]
Report Year:	2007
Report Title:	Fluopicolide - Evaluation of the oral bioavailability of fluopicolide in the rat
Report No:	M-287367-01-1
Document No:	M-287367-01-1
Guideline(s) followed in study:	not applicable
Deviations from current test guideline:	not applicable
Previous evaluation:	yes, evaluated and accepted Addendum 1 to the DAR (2007)
GLP/Officially recognised testing facilities:	not applicable
Acceptability/Reliability:	Yes

Executive summary

In the DAR (2006) the RMS used an estimate for oral absorption of 62%, from the pyridyl radiolabel in the biliary excretion study. It was stated that the basis for using this estimate, rather than that for the phenyl radiolabel (80%) was unclear and hence the more conservative estimate was relied upon for derivation of the AOEL. The applicant/notifier had initially proposed in the tier 2 summary a value of 74%. This paper presents a case for concluding a higher bioavailability than 62% for the rat, based on the existing ADE, PK and metabolism data, with biological justification.

The bile excretion results alone provide an underestimation of the oral bioavailability of fluopicolide as a significant level of entero-hepatic recirculation of fluopicolide and/or its metabolites that would normally occur could not be measured. Additionally the fate of the metabolites formed following cleavage of the fluopicolide molecule differed in that PCA and its related metabolites were more likely to be reabsorbed and eliminated via the urine than BAM and its related metabolites, which would be more likely to be reabsorbed and eliminated via the bile. The blood kinetic data suggest that the systemic exposure was the same for the two radiolabels (functioning entero-hepatic recirculation), whereas the bile excretion study data suggest that there was a difference.

When the entero-hepatic recirculation is taken into account the oral bioavailability of the two radiolabels was found to be closer than originally presumed, with an overall mean range between 75% and 88% of dose. Therefore, the bioavailability value of 74% proposed by the notifier in the tier 2 summary was already a conservative estimate.

What the existing data have indicated

Determination of the oral absorption level is generally obtained from ADE studies using bile duct cannulated rats. This model permits determination of the proportion of the dose that was absorbed and subsequently eliminated *via* the bile. The proportion of the administered radioactivity that is found in the bile, urine (plus cage rinse) and tissues (excluding intestinal contents) is summed to provide the oral absorption value in terms of percentage of dose administered. For fluopicolide two such studies[1,2] were performed using either [Phenyl-U-14C] or [Pyridyl-2,6-14C] radiolabels. The table below presents the results from the single oral low dose groups from these studies.

Table 5.1.1- 60:Recovery of radioactivity from bile duct cannulated rats following single oral dose of [phenyl -U-^{14}C]-fluopicolide or [pyridyl-2,6-14C]-fluopicolide at 10 mg/kg bw (Normalized, expressed as % of administered dose, Mean ± SD, n=4)

	Males		Females	
	Pyridyl label	Phenyl label	Pyridyl label	Phenyl label
Urine	5.84 ± 2.41	4.58 ± 4.24	10.11 ± 4.94	6.61 ± 1.96
Faeces	40.56 ± 6.78	21.62 ± 7.04	38.15 ± 6.16	18.90 ± 6.88
Bile	52.09 ± 9.52	71.12 ± 8.63	49.72 ± 10.11	72.26 ± 6.95
Cage wash	0.70 ± 0.37	0.76 ± 0.78	1.53 ± 1.88	0.89 ± 0.58
Tissues[a]	0.80 ± 0.38	1.93 ± 0.34	0.50 ± 0.13	1.35 ± 0.12
Total absorbed[b]	59.44 ± 6.78	78.38 ± 7.04	61.85 ± 6.16	81.10 ± 6.88
Total	100.0	100.0	100.0	100.0

a Excluding intestinal and stomach contents
b Sum of radioactivity in urine, cage rinse, bile and tissues

Comparison of these recovery normalised results reveals that the principal differences between the two radiolabels appear in the levels observed in the bile and the faeces, with the urinary levels being similar. Overall, the mean total absorbed for the pyridyl radiolabel was 61% and that for the phenyl radiolabel was 80%.

Another measure of systemic exposure can be obtained from the blood and plasma pharmacokinetic data. Studies were again performed using both radiolabels following single oral low doses of 10 mg/kg bw[3]. A summary of these parameters is provided in the table below (taken from the study summary in this document).

[1] [redacted] [Phenyl-U-^{14}C]-AE C638206: Rat Bile Excretion Study, Report N° SA 01383, Document N°: C021984, Edition N°: M-212243-01-1, March 2002 (KCA 5.1.1/04)
[2] [redacted] [Pyridyl-2,6-^{14}C]-AE C638206: Single Oral Low Dose Rat Bile Excretion Study, Report N° SA 02157, Document N°: C032181, Edition N°: M-230976-01-1, February 2003 (KCA 5.1.1/05)
[3] [redacted] [Phenyl-U-14C]-AE C638206 and [Pyridyl-2,6-14C]-AE C638206: Rat Blood and Plasma Kinetics Study, Report N° SA 02012, Document N°: C036987, Edition N°: M-221902-01-1, September 2003 (KCA 5.1.1/06)

Table 5.1.1- 61:Calculated pharmacokinetic parameters following single oral dose of [phenyl -U-^{14}C]-fluopicolide or [pyridyl-2,6-14C]-fluopicolide at 10 mg/kg bw

	Phenyl label				Pyridyl label			
	Blood		Plasma		Blood		Plasma	
	Males	Females	Males	Females	Males	Females	Males	Females
C_{max} (µg equ/g)	1.50 ± 0.24	1.19 ± 0.44	2.20 ± 0.39	1.61 ± 0.67	1.49 ± 0.51	1.18 ± 0.26	2.14 ± 0.62	1.59 ± 0.23
T_{max} (h)	7.5 ± 1	5.5 ± 2.5	8 ± 0	6.5 ± 3	7 ± 1.2	6 ± 1.6	7 ± 1.2	6.5 ± 1.9
$t_{0.5}$ (h)	56.63 ± 1.61	120.7 ± 26.2	18.85 ± 1.49	19.72 ± 6.21	80.34 ± 14.3	140.3 ± 25.4	14.44 ± 2.62	12.67 ± 2.76
$AUC_{(0\text{-}168h)}$ µg.h/g	48.04 ± 8.35	52.87 ± 8.16	54.24 ± 10.9	38.88 ± 9.84	40.59 ± 15.2	45.22 ± 4.78	48.39 ± 20.3	30.61 ± 5.17
$AUC_{(0\text{-}inf)}$ µg.h/g	51.65 ± 8.66	73.54 ± 12.6	55.22 ± 11.4	40.28 ± 8.97	45.37 ± 15.2	67.72 ± 12.9	48.93 ± 20.3	30.96 ± 5.17

C_{max} = maximal concentration, T_{max} = time of maximal concentration, $t_{0.5}$ = terminal elimination half-life, AUC = area under the curve

The AUC values indicated a similar level of systemic exposure for both males and females, in terms of either whole blood or plasma, with no significant differences between the two labels.

The results demonstrated that, following a single oral dose of either label, the general pharmacokinetic profiles were similar for both labels and both sexes. Fluopicolide was absorbed moderately rapidly, followed by a moderately rapid elimination with a slower terminal elimination phase. These results for absorption, in terms of comparison between the labels, are apparently inconsistent with the bile excretion study results.

Other ADE results are available from rats that were not bile duct cannulated[4] [5]. The table below presents dose normalized results from these experiments.

Table 5.1.1- 62:Recovery of radioactivity from tissues and excreta of rats following single oral dose of [phenyl -U-^{14}C]-fluopicolide or [pyridyl-2,6-14C]-fluopicolide at 10 mg/kg bw (Normalized, expressed as % of administered dose, Mean ± SD, n=4)

	Males		Females	
	Pyridyl label	Phenyl label	Pyridyl label	Phenyl label
Urine	20.05 ± 1.24	10.83 ± 3.88	22.33 ± 5.04	13.28 ± 2.93
Faeces	77.11 ± 1.63	86.85 ± 4.31	71.71 ± 4.19	83.82 ± 2.70
Cage wash	2.13 ± 0.99	1.01 ± 0.48	5.48 ± 1.22	1.89 ± 0.60
Tissues	0.71 ± 0.42	1.31 ± 0.08	0.48 ± 0.03	1.01 ± 0.04
Total	100.0	100.0	100.0	100.0

[4] [redacted] [Phenyl-U-14C]-AE C638206: single High and Low Dose Rat A.D.E. Study, Report N° SA 00398, Document N°: C017703, Edition N°: M-204781-01-1, July 2001 (KCA 5.1.1/02)

[5] [redacted].: [Pyridyl-2,6-14C]-AE C638206: Single Oral Low Dose Rat A.D.E. Study, Report N° SA 00477, Document N°: C012989, Edition N°: M-202609-02-1, March 2001 (KCA 5.1.1/03)

There was a tendency towards higher urinary excretion for the pyridyl label, compared to the phenyl label, mirrored by a tendency for lower faecal elimination for the pyridyl label. There was also a tendency for lower tissue levels of the pyridyl label. This all suggests that a proportion of the metabolites differed between the two radiolabels, to such an extent that the elimination profile was altered.

Investigations of metabolism of fluopicolide in the rat[6] [7] demonstrated extensive metabolism, including aromatic ring hydroxylation, hydrolysis, dealkylation, acetylation, oxidative N-dealkylation and conjugation with glucuronic acid, sulphate, and glutathione. The glutathione conjugates were seen to be further metabolised by loss of glycine and glutamic acid to leave cysteine conjugates. The cysteine conjugates were seen to be further metabolised by acetylation to form the mercapturic acids or to be dealkylated and S-methylated to form S-methyl metabolites. The S-methyl metabolites were seen to be oxidised to both sulphones and sulphoxides. These studies demonstrated that the molecule was subject to cleavage leading to the formation of PCA (M-02) and BAM (M-01) to a small, but appreciable extent (up to approximately 10% of the administered dose). The proposed metabolic pathway for fluopicolide in the rat is shown in the Figure at the end of this paper.

Structural identification results from the 10 mg/kg bw pyridyl label indicated that metabolites resulting from cleavage to form PCA accounted for 10% and 2% of administered dose in males and females respectively and were unique to urine. In total, 93% of urine radioactivity in males was assigned a structure that accounted for 16.2% of administered dose, while in females 83% of urine radioactivity was assigned a structure accounting for 17.1% of administered dose. The presence of PCA (M-02) in faeces was suggested by comparison of the metabolite fraction HPLC retention time with that of PCA standard, but this was not confirmed by structural analysis. This would have added a further 2.3% for the males and 0.6% for the females. Summing the components with assigned structures from the urine and faeces, a total of 87.2% of administered radioactivity were identified for males and 90.8% for the females.

Structural identification from the 10 mg/kg bw phenyl label indicated that metabolites resulting from cleavage to form BAM (M-01) accounted for 0.8% and 0.7% of administered dose in males and females respectively and were found in both urine and faeces. The identified structures in urine and faeces accounted for a total of 79.2% of administered radioactivity for males and 75% for the females.

Discussion

Normalized results from the bile excretion studies indicated that the principal difference between the pyridyl and the phenyl labels is in the levels excreted in the bile and the faeces, while urinary levels are similar for both. The calculated means from these studies for total absorbed was 61% for the pyridyl label and 80% for the phenyl label.

Meanwhile, the blood plasma pharmacokinetic data indicated general similarity between the labels and the sexes, including comparable exposure at approximately 60%.

A comparison of recoveries from the bile excretion studies with those from the non-cannulated studies is shown in the following table.

[6] [redacted] [Phenyl-U-14C]-AE C638206: Rat Metabolism Following Administration of a Single Oral Low Dose, Report N° SA 00581, Document N°: C039583, Edition N°: M-227026-02-2, March 2004 (KCA 5.1.1/11)

[7] [redacted] [Pyridyl-2,6-14C]-AE C638206: Rat metabolism following administration of a single oral low dose, Report N° SA 00550, Document N°: C039580, Edition N°: M-227023-02-2, February 2004 (KCA 5.1.1/09)

Table 5.1.1- 63: Comparison: Recoveries of radioactivity from tissues and excreta of rats following single oral dose of [phenyl -U-^{14}C]-fluopicolide or [pyridyl-2,6-14C]-fluopicolide at 10 mg/kg bw (Normalized, expressed as % of administered dose)

	Males				Females			
	Cannulated		Non-cannulated		Cannulated		Non-cannulated	
	Pyridyl	Phenyl	Pyridyl	Phenyl	Pyridyl	Phenyl	Pyridyl	Phenyl
Urine + cagewash	6.5	5.3	22.2	11.8	11.6	7.5	27.8	15.2
Faeces+ bile	92.7	92.7	77.1	86.9	87.9	91.2	71.7	83.8
Tissues	0.8	1.9	0.7	1.3	0.5	1.4	0.5	1.0
Total	100	100	100	100	100	100	100	100

The data from non-cannulated rats indicate higher levels of radioactivity in the urine with the pyridyl label, as opposed to higher levels in the faeces with the phenyl label (*ca* 10-12% difference). The inference from this, in context with the blood/plasma kinetic data, is that the degree of absorption/elimination was similar between the two radiolabels, but with a preference for the urinary route of elimination with the pyridyl label and for the biliary route with the phenyl label.

With bile duct cannulation, the difference in urinary elimination between labels was reduced from 10-12% to *ca* 1-4% of dose. Meanwhile, the difference between labels for biliary excretion represented *ca* 20% of dose, the phenyl label being at *ca* 50% and the pyridyl label at *ca* 70% (from the first table above).

A key observation is the decrease in urinary elimination following bile duct cannulation, implying that there is active entero-hepatic recirculation. Removal of the bile content from the amount in the intestine led to its not being reabsorbed. The pyridyl label appears to have been more susceptible to this than the phenyl label.

Comparison of the urinary elimination data for cannulated versus non-cannulated rats for the phenyl label shows that the reduction was 6.5-7.7%. The same comparison for the faeces of non-cannulated rats versus the sum of faecal and biliary elimination for the cannulated rats shows that the radioactivity was 5.9% higher for cannulated males and 7.3% higher for cannulated females. This close match indicates that it is highly likely that this 6-7% of administered dose represented the radioactivity that would have been reabsorbed and eliminated in the urine of non-cannulated rats.

The same calculations for the pyridyl label indicate a reduction in urinary elimination of 15.6-16.2% for cannulated rats, which corresponds to an increase in faecal + biliary elimination of 15.5-16.2% of dose. Thus, it would appear that entero-hepatic recirculation of the pyridyl radiolabel was greater than for the phenyl label. This difference must relate to a difference in metabolic fate involving cleavage of the fluopicolide molecule. The metabolism studies indicate that up to at least *ca* 10% of administered dose could be subject to cleavage, based on detection of cleavage products (PCA, BAM and/or their metabolites).

Studies performed with single oral doses of either PCA[8] or BAM[9] demonstrated that their ADME profiles differed. Following administration of PCA at the rate of 10 mg/kg bw the majority (>86%) of the radioactivity was eliminated *via* the urine with approximately 7% dose found in the faeces. The PCA parent molecule accounted for between 98% and 99% of the radioactivity in the urine and 97% to 98% of the radioactivity found in the faeces indicating that it underwent very little biotransformation and was very well absorbed from the intestine. Following administration of BAM at the same rate of 10 mg/kg bw the majority (>81%) of the radioactivity was eliminated *via* the urine with approximately 13% dose found in the faeces. The BAM parent molecule accounted for approximately 17% of the radioactivity in the urine and 84% to 89% of the radioactivity found in the faeces indicating that BAM was subject to a greater degree of biotransformation than PCA, including aromatic ring hydroxylation, hydrolysis, dealkylation, acetylation, and conjugation with glucuronic acid, sulphate and glutathione (similar to that observed for fluopicolide) and was eliminated to a greater extent *via* the bile.

Thus, the difference between the two radiolabels could be due to the formation of cleaved metabolites PCA and BAM, whose fates differed in that a much greater proportion of the PCA derived metabolites would be reabsorbed and eliminated *via* the urine than the BAM derived metabolites. It is therefore reasonable to calculate the bioavailability of fluopicolide based on the results of both the cannulated and non-cannulated rats, using the urinary data from the non-cannulated rats to take into account the material undergoing entero-hepatic recirculation and the biliary elimination data from the cannulated rats (tissue data was taken from the non-cannulated rats). This yields mean percentages for the pyridyl radiolabel of 75% and 78% of dose for the males and females respectively. The mean percentage bioavailability derived from the phenyl radiolabel was 84% and 88% for the males and females respectively.

Conclusions

Bile excretion results alone provide an underestimation of the oral bioavailability of fluopicolide, as a significant level of entero-hepatic recirculation of fluopicolide and/or its metabolites is apparent and was not measured. In addition, the fate of metabolites formed following cleavage of the fluopicolide molecule differed, in that PCA and its metabolites were more likely to be reabsorbed and eliminated in the urine than BAM and its metabolites, which would be more likely to be eliminated in the bile. It is for this reason that the blood kinetic data suggest that systemic exposure was the same for the two radiolabels (in the presence of functioning entero-hepatic recirculation), while the bile excretion study data suggested that there was a difference.

When entero-hepatic recirculation is taken into account, the oral bioavailability of the two radiolabels was found to be closer than originally presumed, with an overall range of 75% to 88% of dose.

The bioavailability value of 74% proposed by the applicant notifier in the tier 2 summary was therefore already a conservative estimate.

[8] [redacted] [Pyridyl-2,6-14C]-AE C657188 (PCA): Single Oral Low Dose Rat A.D.M.E. Study, report N° SA 01093, Document N°: C024615, Edition N°: M-217250-01-1, June 2002

[9] [redacted] [Phenyl-U-14C]-AE C653711 (BAM): Single Oral Low Dose A.D.M.E. Study in the Rat, report N° SA 02156, Document N°: C035245, Edition N°: M-218350-01-1, July 2003

Figure 5.1.1-14: Proposed metabolic pathway for fluopicolide in the rat, Part 1 of 3

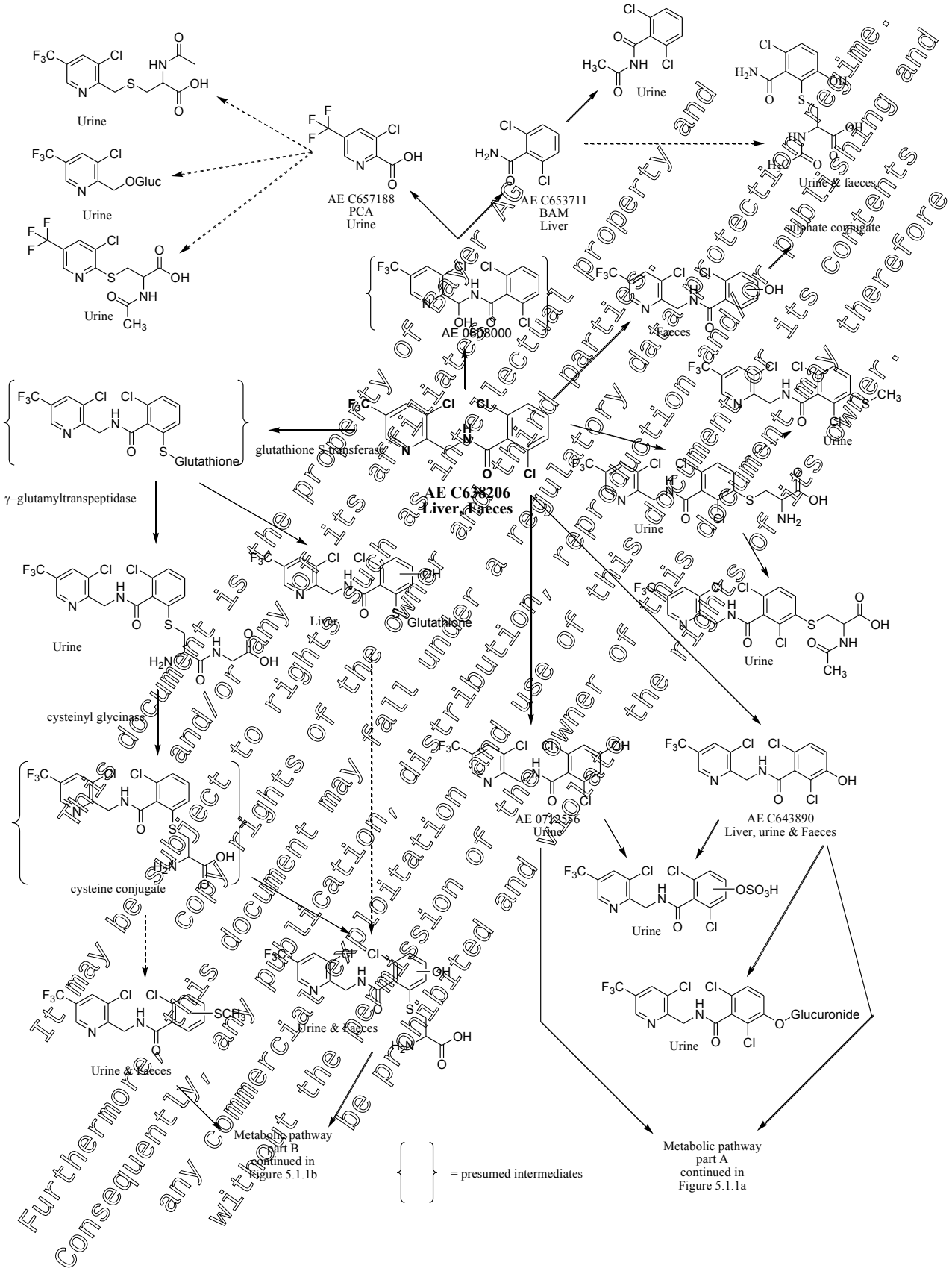


Figure 5.1.1-15: Proposed metabolic pathway for fluopicolide in the rat, Part 2 of 3

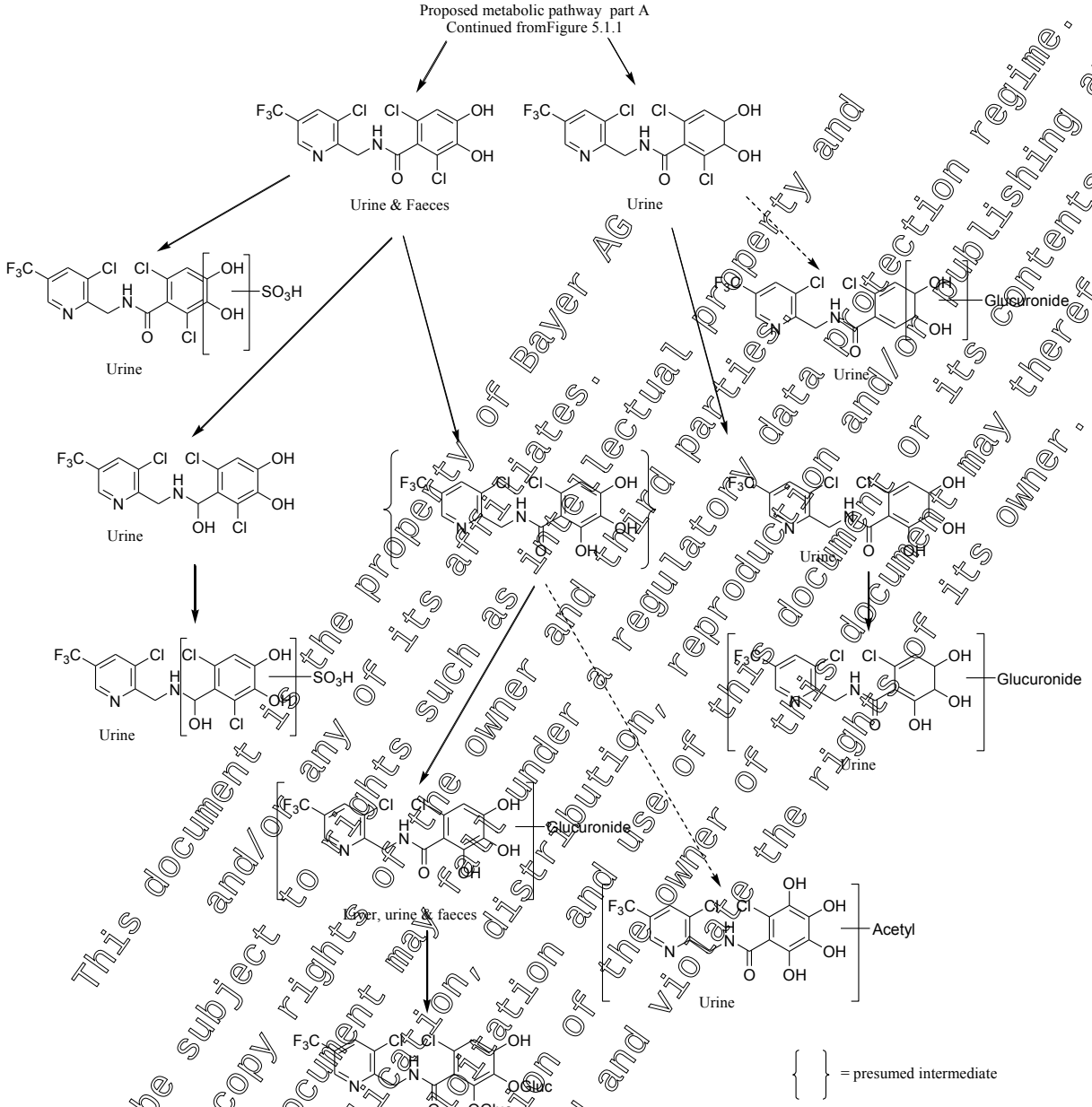
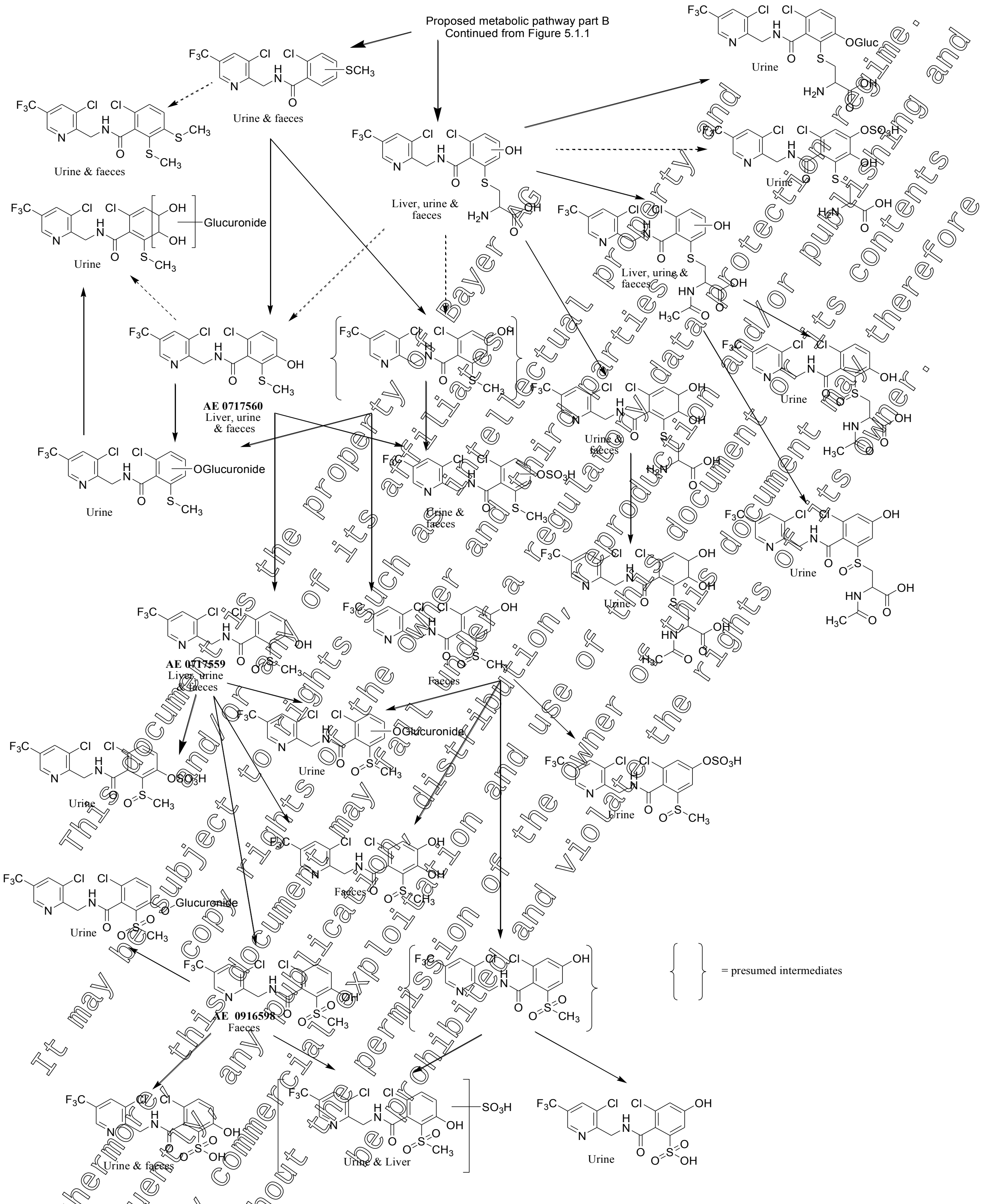


Figure 5.1.1-16: Proposed metabolic pathway for fluopicolide in the rat, Part 3 of 3



Assessment and conclusion by applicant:

An acceptable and valid argument for adoption of a bioavailability value of at least 74% of dose.

Data Point:	KCA 5.1.1/14
Report Author:	[redacted]
Report Year:	2003
Report Title:	(Phenyl-U-14C)-AE C653711 (BAM): Single oral high dose A.D.M.E. study in the rat
Report No:	C035247
Document No:	M-218352-01-1
Guideline(s) followed in study:	EU (=EEC): 94/79/EC, 1994; JMAF: 12, Nohsan No. 8147, 200; USEPA (=EPA): OPPTS 870.7485, 1998
Deviations from current test guideline:	None
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

The absorption, distribution, excretion, and metabolism of [phenyl-UL-^{14}C]-M-01 (referred to as AE C653711 in the report) was investigated in male and female Sprague Dawley rats following a single oral dose level of 150 mg/kg bw.

[Phenyl-UL-^{14}C]-M-01 was administered orally to two groups of four male or female rats at a high dose of 150 mg/kg bw. Urine and faeces samples were collected for seven days until sacrifice, when organs, GIT, skin and carcass were collected.

Mean recoveries were 92% of the administered dose for male rats and 98% for female rats. Oral absorption was 80% of dose for male rats and 86% for females as calculated from the sum of radioactivity in urine, cage washes and tissues.

The routes and rates of elimination were very similar between male and female rats. Excretion of radioactivity was relatively slow, with 96 hours required for close to complete elimination of the radioactivity via the urine. 91 and 97% of the administered dose had been excreted in urine, faeces, or cage wash, with ≤ 1.2% of the dose remaining in the body. At sacrifice (168 hours), rats had excreted 79 to 84% of the administered dose in the urine plus cage washes and 12 to 13% with the faeces. Highest tissue residues were found in the skin & fur at 3.783 µg equiv./g and 5.081µg equiv./g for male and female rats respectively. The next most significant residues were seen in liver and kidney with mean values of 2.083 and 2.987 µg equivalents/g respectively in male rats. A similar distribution pattern was seen in female rats, where the mean levels of radioactivity in liver and kidney were 2.257 and 2.791 µg equivalents/g respectively.

[Phenyl-UL-^{14}C]-M-01 was intensively metabolised with up to 20 radioactive components detected in urine and 12 in the faecal extracts. Fifteen metabolites, plus the test item, were identified in urine and faeces. The proposed biotransformation pathway of M-01 in the rat is shown in Figure 5.1.1- 17.

Unchanged M-01 was identified in urine and faecal samples from male and female rats, totalling 13 to 25% of the dose.

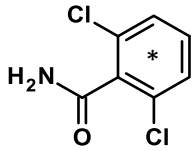
Biotransformations observed included aromatic ring hydroxylation, hydrolysis, decarboxylation, acetylation, and conjugation with glucuronic acid, sulfate and glutathione. Glutathione conjugates were further metabolised by loss of glycine and glutamic acid to leave cysteine conjugates. The cysteine conjugates were further metabolised by acetylation to form the mercapturic acids or to dealkylated and S-methylated to form S-methyl metabolites. The principal metabolic pathway was formation of a mercapturic acid conjugate of hydroxy-chlorobenzamide, present in urine at 18-21% of dose (USHD/9) and in faeces at <1% (FSHD/4).

I. Materials and methods

A. Materials

1. Test material: [Phenyl-U-^{14}C]-M-01 (referred to as AE C653711 in the report)

Chemical structure:



* labelling position

Batch no.:	SEL/1059
Specific radioactivity:	Initial : 2.246 GBq/mmole (60.9 mCi/mmole) Final: 5.4825 MBq/mmole (0.7790 µCi/mg)
Radiochemical purity:	98.49% (HPLC)

2. Vehicle: 0.75% methyl-cellulose in water

3. Test animals:

Species:	Rat *(Rattus norvegicus domesticus)*
Strain:	Sprague-Dawley CD
Age:	6 to 10 weeks
Weight at dosing:	228 to 233 g (males) and 202 to 211 g (females)
Source:	[redacted]
Acclimation period:	At least 5 days
Identification:	Animals were identified by ear tattoo
Diet:	Certified rodent diet A04C, obtained from UAR, Villemoisson, France The rodent diet was removed approximately 18 hours before administration of the radiolabelled test material and replaced one hour post dosing.
Water:	Filtered and softened water obtained from the municipal supply that was routinely analysed to ensure that no contaminants were present that could affect the outcome of the study
Housing:	Animals were kept individually in Jencon's Metabowls Mk III metabolism units
Environmental conditions:	
Temperature:	22 ± 2°C
Humidity:	55 ± 15%.
Ventilation:	Average of 15 air changes per hour
Photoperiod:	12-hour light & 12 hour dark.

4. Preparation of dosing solutions

A stock solution radiolabelled M-01 (SEL/1059) dissolved in acetonitrile was mixed with non-radiolabelled test material (Batch number R001724) to obtain a homogeneous solution. For each test, an adequate portion of the solution was evaporated under a gentle stream of nitrogen and the residue resuspended in 0.75% methyl-cellulose in water.

B. Study design and methods

1. Dose regimen and design of tests

Test no.	Administered single dose of ^{14}C-M-01, route (experiment)	^{14}C-label	Number of rats and sex	Collection of samples during the test and at sacrifice	Duration
1	150 mg/kg bw, oral (single high dose)	phenyl-UL	4 males	urine, faeces, blood, plasma, organs, GIT, skin, carcass	168 h
2	150 mg/kg bw, oral (single high dose)	phenyl-UL	4 females	urine, faeces, blood, plasma, organs, GIT, skin, carcass	168 h

The rats were given a single dose of radiolabelled [phenyl-UL-^{14}C]-M-01 uniformly labelled in the aromatic ring.

[Phenyl-UL-^{14}C]-M-01 was administered orally to groups of four male or female rats at a high dose of 150 mg/kg bw. Urine, cage washes and faeces were collected for seven days until sacrifice, when blood, plasma, organs, GIT, skin and carcass were also collected.

2. Dosing

Suspensions of the test compound in 0.75% methyl-cellulose in water were administered orally to each rat. The test suspension was administered by gavage at a rate of 5g dose suspension per kg of rat body weight.

The dose suspension was assayed for test item concentration (by HPLC) and for radioactivity content (by LSC) at three time points (before, mid-way through and after the dosing procedure). Actual dose levels were close to nominal levels:

Test ID	Nominal Dose (mg/kg bw)	Actual Dose (mg/kg bw)
1	150	141.71
2	150	141.84

3. Collection of excreta

After the administration of the radiolabelled test substance, the rats were kept individually in metabowl units which allowed for separate and quantitative collection of urine and faeces.

Urine was collected separately for each rat in a cryogenic trap cooled with dry ice at the following intervals after administration of the radiolabelled dose:

6, 24, 48, 72, 96, 120, 144 and 168 hours in tests 1 and 2

Metabowl cages were washed with distilled water every 24 hours and with acetonitrile after the animals had been removed from the units at the end of the in-life phase.

The radioactivity in urine and metabowl rinses was determined by LSC.

Faeces were collected separately for each rat every 24 hours after administration of the radiolabelled dose. Faeces samples were homogenised with approximately twice their volume of water. The radioactivity was determined by combustion followed by LSC.

4. Expired air

The collection of expired carbon dioxide and other volatiles was not undertaken in this study as it had been established in the single oral low dose study (M-218350-01-1) that quantitative mass balances were obtained without collection of expired air.

5. Sacrifice

The rats were anaesthetized by injection of Imalgene-500 and sacrificed by exsanguination.

6. Blood, tissues, and organs at sacrifice

At sacrifice, cardiac blood was collected, and plasma prepared by centrifugation. The radioactivity in blood was determined by combustion and in plasma by LSC directly.

The following organs and tissues were collected:

- liver, kidneys, heart, lungs, brain, cardiac blood, spleen, pancreas, muscle, abdominal fat, ovaries (for females), testes (for males), stomach plus contents, intestine plus content, bone and marrow, adrenals, the skin & fur, uterus (for females), eyes, Harderian glands and thyroids

The residual carcass was also retained for analysis.

Fat, testes, bone (plus marrow), uterus, as well as whole tissues such as ovaries, eyes, Harderian glands, thyroids, adrenals were minced by hand with scissors. Other tissues were homogenised using an Ultra-Turrax with small amounts of water when necessary. The radioactivity was determined by combustion followed by LSC

The skin & fur, uterus and carcass samples were solubilised in alcoholic 2M KOH for 24 hours at 50°C. The radioactivity of the solubilised material was determined by LSC.

7. Sample handling and storage

Whenever possible samples were processed as they were collected. Remaining samples and samples awaiting processing were stored at -20°C in the dark until required.

8. Preparation of samples for analysis

Urine and faecal samples contained almost the entire amount of the recovered radioactivity and therefore, the metabolism study was performed with these two categories of samples. Samples were combined to representative pools for each test group.

Pools of urine samples were prepared to represent the following excretion intervals:

0 – 6, 6 – 24, 24 – 48, 48 – 72, 72 – 96 and 96 – 120 hours for test 1 and 2

Urine samples were concentrated prior to analysis by HPLC. Concentrated urine samples from 0 – 6 hours and 6 - 24 hours were combined and analysed as a single pooled sample for quantification of urine metabolites.

Pools of faeces samples were prepared to represent the following excretion intervals:

24 - 48 and 48 – 72 hours for test 1 and 2

The chromatographic profiles of 0-24 h faecal samples were also investigated but the results were not reported thus, the low levels of radioactivity led to poor chromatograms.

Faecal samples were centrifuged for 20 minutes, the aqueous supernatant decanted and the remaining pellet extracted twice with acetonitrile. All three extracts were pooled and concentrated prior to analysis by HPLC.

Radioactivity in extracts was determined by LSC and in the remaining solids by combustion followed by LSC.

9. Analytical methods

The chromatographic separation and quantification of the metabolites present in urine and faecal samples was performed using the High Performance Liquid Chromatography (HPLC) with radiodetection and by LC-MS/MS methods.

II. Results and Discussion

A. Recovery

The mean recovery over a seven-day period post-administration was found to be 92.21% in male rats and 98.18% in female rats. A summary of the radioactivity as percent of the administered dose found in urine, faeces and in organs and tissues at sacrifice is presented in table 5.1.1-64.

Table 5.1.1- 64: Recovery of radioactivity after administration of [phenyl-UL-^{14}C]-M-01 (BAM) at rate of 150 mg/kg b/w

Percent of administered dose (mean values)

Sample	Test 1 male oral 150 mg/kg bw		Test 2 female oral 150 mg/kg bw	
	Mean	**SD**	**Mean**	**SD**
Urine	69.28	1.74	78.14	1.74
Cage wash	9.33	3.53	6.20	1.58
Faeces	12.44	3.50	12.63	1.11
Tissues	1.17	0.27	1.21	0.21
Total	**92.21**	**1.46**	**98.18**	**1.42**

SD = standard deviation

The major route of excretion was *via* the urine for both sexes with a mean of 78.61% of the administered dose in male rats and 84.34% in female rats eliminated (urine plus cage washes) during the 7-day sampling period. The proportion of radioactivity eliminated *via* the faecal route was 12.44% in male rats and 12.63% in female rats.

B. Absorption

The estimated minimum levels of absorption were measured as the total radioactivity in urine, cage washes plus tissues. The results showed that at least 80% of the administered dose was absorbed by male rats and similar results were seen in female rats with 86% of dose absorbed. Therefore, high oral bioavailability was seen in both male and female rats (see table 5.1.1-64).

C. Distribution

Levels of radioactivity remaining in tissues at 168 hours represented a sum total of 1.17% of the administered dose in male rats and 1.21% in female rats. The distribution of radioactive residues in the rat are summarised in table 5.1.1-65 expressed as parent equivalent concentrations in µg/g.

The highest tissue concentrations in male and female rats were found in skin & fur with mean values of 3.783 and 5.081 µg equivalents/g respectively.

In the case of male rats, the second most significant residues were seen in liver and kidney with mean values of 2.083 and 2.987 µg equivalents/g respectively. The levels of radioactivity in adrenals, harderian glands and intestine & contents of male rats presented mean values of 1.585, 1.107 and 1.042 µg equivalents/g respectively. The remaining tissue concentrations, in male rats, were below 1.000 µg equivalents/g.

A similar distribution pattern was seen in female rats, where the mean levels of radioactivity in liver and kidney were 2.257 and 2.791 µg equivalents/g respectively. The levels of radioactivity in adrenals, harderian glands and intestine & contents of female rats presented mean values of 1.604, 1.335 and 1.101 µg equivalents/g respectively. The remaining tissue concentrations, in female rats, were also below 1.000 µg equivalents/g.

Table 5.1.1- 65: Concentration of [phenyl-UL-^{14}C]-M-01 (BAM) residues in rat tissues following a single oral dose at 150 mg/kg bw

µg M-01 equivalents/g tissue

Organs/ Tissues	Test 1 oral male 150 mg/kg bw		Test 2 oral female 150 mg/kg bw	
	Mean	SD	Mean	SD
Cardiac blood	0.66	0.217	0.791	0.251
Intestine & contents	1.042	0.454	1.101	0.303
Harder's Gland	1.107	0.263	1.335	0.321
Residual Carcass	0.663	0.159	0.701	0.125
Skin & Fur	3.783	0.791	5.081	0.959
Cardiac Plasma	0.558	0.215	0.587	0.227
Eyes	0.517	0.196	0.663	0.232
Brain	0.601	0.232	0.616	0.275
Fat	0.4	0.133	0.376	0.099
Heart	0.742	0.228	0.755	0.266
Lungs	0.751	0.245	0.85	0.27
Spleen	0.8	0.245	0.83	0.233
Liver	2.083	0.689	2.257	0.413
Kidneys	2.987	0.713	2.791	0.645
Stomach & contents	0.508	0.235	0.621	0.228
Thyroids	n.d.	n.a.	n.d.	n.a.
Testes	0.658	0.256	n.a.	n.a.
Ovaries	n.d.	n.a.	0.904	0.267
Pancreas	0.671	0.237	0.718	0.237
Adrenal	1.585	0.414	1.604	0.426
Uterus	n.a.	n.a.	0.596	0.22
Muscle	0.675	0.207	0.695	0.22
Bone & Marrow	0.391	0.151	0.386	0.157

SD = standard deviation, n.d. = not detected, n.a. = not applicable

D. Excretion

The rate of elimination was slow with 96 hours being necessary for the excretion of at least 90% of the radioactivity eliminated in the urine. The routes and rates of elimination were very similar between male and female rats. These data are presented in table 5.1.1-66.

Table 5.1.1- 66: Elimination of radioactivity after administration of [phenyl-UL-^{14}C]-M-01 (BAM) at rate of 150 mg/kg b/w

Percent of administered dose (mean values)

Sample	Time [h post admin.]	Test 1 male oral 150 mg/kg bw		Test 2 female oral 150 mg/kg bw	
		Mean	SD	Mean	SD
Urine	0-6	3.12	0.27	4.09	1.08
	6-24	23.88	2.26	23.81	1.08
	24 -48	48.80	3.14	51.14	2.53
	48 -72	60.13	2.12	65.77	2.53
	72-96	65.02	2.20	72.84	2.61
	96-120	67.60	2.10	76.20	1.93
	120-144	68.75	1.86	77.55	1.77
	144-168	69.28	1.74	78.14	1.74
Faeces	0-24	3.20	1.74	0.77	0.54
	24 -48	8.00	3.25	7.48	1.06
	48 -72	10.44	3.24	10.40	1.40
	72-96	11.32	3.49	11.53	1.17
	96-120	11.83	3.46	12.24	1.16
	120-144	12.29	3.48	12.52	1.13
	144-168	12.44	3.50	12.63	1.11
Cage wash	0-168	9.33	2.53	6.20	1.58
Total eliminated		**91.05**	**1.29**	**96.97**	**1.47**

SD = standard deviation

E. Metabolism

The unchanged test item, M-01, was one of the major components in urine and faeces reaching a sum total of 13.0% in males and 24.60% in females (7.65% in male urine, 17.85% in female urine, 5.35% in male faeces and 6.75% in female faeces).

Several different metabolic pathways were postulated for the biotransformation of M-01. One of them was the hydrolysis of the test product leading to the dichlorobenzoic acid (AE C416656) which accounted for 0.64% dose in male rats and 0.65% in female rats. Hydrolysis of the aryl amide functional group was also seen. Metabolites USHD/4 (O-glucuronide conjugate) and USHD/10a (sulphate conjugate) were transformed via carboxylic acid derivative intermediates which were then subsequently decarboxylated leading to USHD/15 (O-glucuronide conjugate of dichlorophenol) and USHD/12 (O-sulphate conjugate of dichlorophenol) respectively.

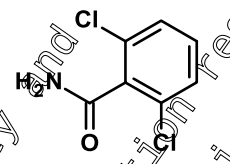
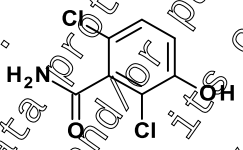
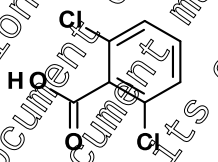
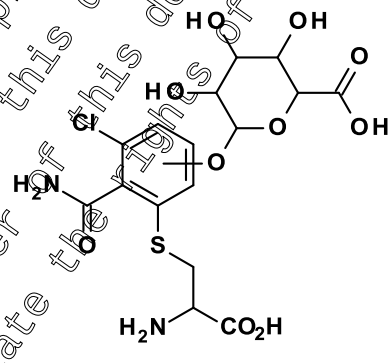
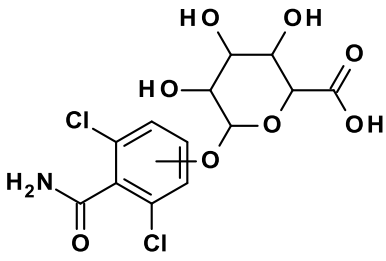
The principal metabolic pathway led to USHD/9, USHD/16 and FSHD/4 identified as mercapturic acid conjugates of hydroxy-chlorobenzamide. The major metabolite eliminated in the urine was USHD/9 which accounted for a sum total of *ca.* 21% dose in male urine and 18% in female urine. The metabolite USHD/16 is an isomer of USHD/9 present in urine samples at lower levels, 0.45% and 0.56% for male and female rats respectively. The faecal metabolite FSHD/4 was the same mercapturic acid conjugate as USHD/9. There were two potential pathways that would lead to the mercapturic acid conjugate of hydroxy-chlorobenzamide, the first was aromatic dehalogenation of M-01 followed by the action of glutathione S-transferase leading to a glutathione conjugate intermediate. The GS-intermediate would have been further bio transformed (losing glutamic acid and glycine) leading to a cysteine conjugate that was subsequently hydroxylated in the aromatic ring, resulting in USHD/6. The metabolite USHD/6 was the cysteine conjugate of hydroxy-chlorobenzamide that was subsequently N-acetylated leading to its derivative mercapturic acid conjugate. It is postulated that the aromatic position of the hydroxyl group and/or the S-cysteine group could have led to isomers (USHD/9 and USHD/16). The second proposed metabolic pathway leading to USHD/9, USHD/16 and FSHD/4 was the aromatic hydroxylation on the test product, that was subsequently dehalogenated and followed by the action of GSH enzymes, loss of glutamic acid and glycine. See the metabolic pathway described below for further details of the two biotransformation pathways leading to the major metabolite (mercapturic acid conjugate).

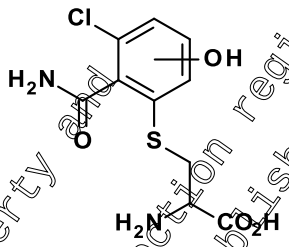
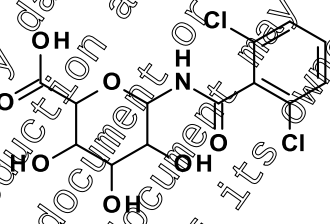
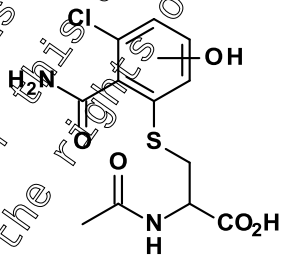
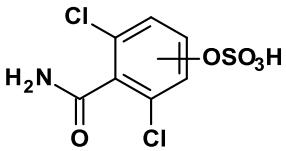
There were at least two different hydroxy derivative metabolites of the test product, one of them USHD/11 found in urine samples, was identified as M-04 (referred to as AE C657378 in the report). The other was an isomer (FSHD/7) seen in faeces. The hydroxyl metabolite was a potential intermediate leading to USHD/9, USHD/16 and FSHD/4, as described above but was also the intermediate leading to O-glucuronide and O-sulphate conjugates from the action of glucuronidase and sulfatase enzymes. A different glucuronide conjugate USHD/8 was formed by the action of N-glucuronide enzymes. The metabolite USHD/8 reached a total of 0.53% in male urine and 0.43% in female urine.

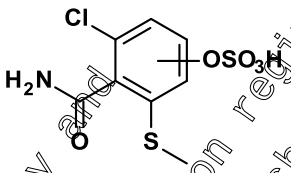
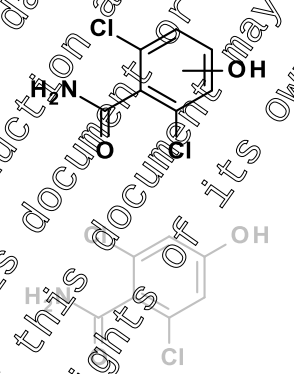
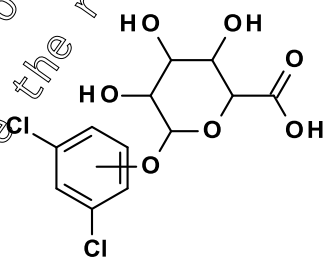
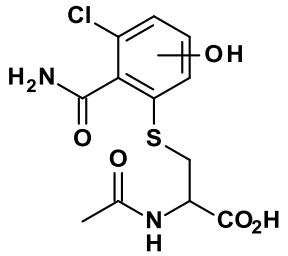
The metabolite USHD/6 accounted for *ca.* 4.52% in male rats and *ca.* 10.76% in female rats. USHD/6 was further metabolised by the action of glucuronide enzymes leading to USHD/3 the O-glucuronide conjugate of USHD/6. The metabolite USHD/3 accounted for a total of 4.97% in male urine and 3.78% in female urine. A different biotransformation of USHD/6 was obtained by the action of S-dealkylation enzymes (aryl cysteine lost) leading to thiomethyl hydroxy-chlorobenzamide (FSHD/8) which was subsequently metabolised by the action of sulfatase enzymes leading to O-sulphate conjugate of thiomethyl-chlorobenzamide (USHD/10b). The thiomethyl hydroxy-chlorobenzamide (FSHD/8) could also have been obtained by hydroxylation of the thiomethyl intermediate (FSHD/10).

The following table presents a summary of the identified metabolites. The levels of M-01 and its metabolites quantified in urine and faeces are provided in Tables 5.1.1-68 and 5.1.1-69 for male and female rats, respectively.

Table 5.1.1- 67: Identified metabolites

Report name (peak ID in the profiles)	Chemical name (IUPAC)	Chemical Structure
Test item M-01 (BAM, BCS-AA65784, AE C653711) peak ID in the profiles: USHD/18 and FSHD/11	2,6-dichlorobenzamide	
M-04 (BCS-AX26226, AE C657378, 3-hydroxy BAM) peak ID in the profiles: USHD/11	2,6-dichloro-3-hydroxybenzamide	
AE C416656 peak ID in the profiles: USHD/7	2,6-dichlorobenzoic acid	
USHD/3 Cysteine and O-glucuronide conjugate of chlorobenzamide	Depending on the position of the glucuronide conjugation IUPAC: 6-[4-(2-amino-2-carboxy-ethyl)sulfanyl-3-carbamoyl-2-chloro-phenoxy]-3,4,5-trihydroxy-tetrahydropyran-2-carboxylic acid Or 6-[3-(2-amino-2-carboxy-ethyl)sulfanyl-4-carbamoyl-5-chloro-phenoxy]-3,4,5-trihydroxy-tetrahydropyran-2-carboxylic acid Or 6-[2-(2-amino-2-carboxy-ethyl)sulfanyl-3-carbamoyl-4-chloro-phenoxy]-3,4,5-trihydroxy-tetrahydropyran-2-carboxylic acid	
USHD/4 O-Glucuronide conjugate of dichlorobenzamide	Depending on the position of the glucuronide conjugation IUPAC: 6-(4-carbamoyl-3,5-dichloro-phenoxy)-3,4,5-trihydroxy-tetrahydropyran-2-carboxylic acid Or 6-(3-carbamoyl-2,4-dichloro-phenoxy)-3,4,5-trihydroxy-tetrahydropyran-2-carboxylic acid	

Report name (peak ID in the profiles)	Chemical name (IUPAC)	Chemical Structure
USHD/6 Cysteine conjugate of hydroxy-chlorobenzamide	Depending on the position of the hydroxylation IUPAC: 2-amino-3-(2-carbamoyl-3-chloro-5-hydroxy-phenyl)sulfanyl-propanoic acid Or 2-amino-3-(2-carbamoyl-3-chloro-6-hydroxy-phenyl)sulfanyl-propanoic acid Or 2-amino-3-(2-carbamoyl-3-chloro-4-hydroxy-phenyl)sulfanyl-propanoic acid	
USHD/8 N-Glucuronide conjugate of dichlorobenzamide	IUPAC: 6-[(2,6-dichlorobenzoyl)amino]-3,4,5-trihydroxy-tetrahydropyran-2-carboxylic acid	
USHD/9 FSHD/4 Mercapturic acid conjugate of hydroxy-chlorobenzamide Isomer of USHD/16	Depending on the position of the hydroxylation IUPAC: 2-acetamido-3-(2-carbamoyl-3-chloro-5-hydroxy-phenyl)sulfanyl-propanoic acid Or 2-acetamido-3-(2-carbamoyl-3-chloro-6-hydroxy-phenyl)sulfanyl-propanoic acid Or 2-acetamido-3-(2-carbamoyl-3-chloro-4-hydroxy-phenyl)sulfanyl-propanoic acid	
USHD/10 a O-Sulfate conjugate of dichlorobenzamide	Depending on the position of the O-sulfate conjugate IUPAC: (4-carbamoyl-3,5-dichloro-phenyl) hydrogen sulfate Or (3-carbamoyl-2,4-dichloro-phenyl) hydrogen sulfate	

Report name (peak ID in the profiles)	Chemical name (IUPAC)	Chemical Structure
USHD/10 b O-Sulfate conjugate of thiomethyl-chlorobenzamide	Depending on the position of the O-sulfate conjugate IUPAC: (4-carbamoyl-3-chloro-5-methylsulfanyl-phenyl) hydrogen sulfate Or (3-carbamoyl-2-chloro-4-methylsulfanyl-phenyl) hydrogen sulfate Or (3-carbamoyl-4-chloro-2-methylsulfanyl-phenyl) hydrogen sulfate	
FSHD/7 Hydroxy-chlorobenzamide Isomer of M-04 (AE C657378)	IUPAC: 2,6-dichloro-4-hydroxy-benzamide Although the position of the hydroxylation was not stated in the report it is presumed the following is the only possibility as 2-hydroxy isomer was identified by co-chromatography with M-04 (AE C657378)	
USHD/15 O-Glucuronide conjugate of dichlorophenyl	Depending on the position of the glucuronide conjugation IUPAC: 6-(2,4-dichlorophenoxy)-3,4,5-trihydroxy-tetrahydropyran-2-carboxylic acid Or 6-(3,5-dichlorophenoxy)-3,4,5-trihydroxy-tetrahydropyran-2-carboxylic acid	
USHD/16 Mercapturic acid conjugate of hydroxy-chlorobenzamide Isomer of USHD/9 & FSHD/4	Depending on the position of the hydroxylation IUPAC: 2-acetamido-3-(2-carbamoyl-3-chloro-5-hydroxy-phenyl)sulfanyl-propanoic acid Or 2-acetamido-3-(2-carbamoyl-3-chloro-6-hydroxy-phenyl)sulfanyl-propanoic acid Or 2-acetamido-3-(2-carbamoyl-3-chloro-4-hydroxy-phenyl)sulfanyl-propanoic acid	

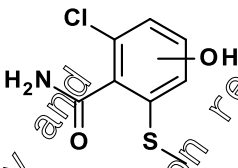
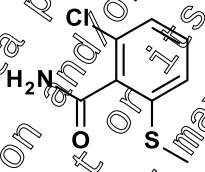
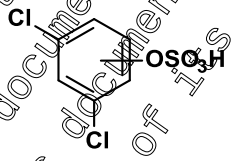
Report name (peak ID in the profiles)	Chemical name (IUPAC)	Chemical Structure
FSHD/8 Thiomethyl-hydroxychlorobenzamide	Depending on the position of the hydroxylation IUPAC: 2-chloro-4-hydroxy-6-methylsulfanyl-benzamide Or 2-chloro-3-hydroxy-6-methylsulfanyl-benzamide Or 6-chloro-3-hydroxy-2-methylsulfanyl-benzamide	
FSHD/10 Thiomethyl-chlorobenzamide	IUPAC: 2-chloro-6-methylsulfanyl-benzamide	
USHD/19 O-Sulfate conjugate of dichlorophenyl	Depending on the position of the O-sulfate conjugate IUPAC: (3,5-dichlorophenyl) hydrogen sulfate Or (2,4-dichlorophenyl) hydrogen sulfate	

Table 5.1.1- 68: Balance of [phenyl-UL-^{14}C]-M-01 and metabolites excreted after oral administration to male rats (expressed as % dose administered)

Peak ID	Name	Test 1 (male, 150 mg/kg bw)		
		Urine (0 - 120 h)	Faeces (0 - 72 h)	Total
		% of dose administered		
USHD/3	Cysteine and O-glucuronide conjugate of chlorobenzamide	4.97	-	4.97
USHD/4	O-glucuronide conjugate of dichlorobenzamide	1.46	-	1.46
USHD/6	Cysteine conjugate of hydroxy-chlorobenzamide	4.52	-	4.52
USHD/7	AE C416656	0.64	-	0.64
USHD/8	N-glucuronide conjugate of dichlorobenzamide	0.53	-	0.53
USHD/9, FSHD/4	Mercapturic acid conjugate of hydroxy-chlorobenzamide	20.85	0.38	21.23
USHD/10	O-sulfate conjugate of dichlorobenzamide O-sulfate conjugate of thiomethyl-chlorobenzamide	13.83	-	13.83
USHD/11	AE C657378	7.32	-	7.32
FSHD/7	Hydroxy-chlorobenzamide	-	0.24	0.24
USHD/15	O-glucuronide conjugate of dichlorophenyl	0.64	-	0.64
USHD/16	Mercapturic acid conjugate of hydroxy-chlorobenzamide	0.45	-	0.45
FSHD/8	Thiomethyl-hydroxychlorobenzamide	-	0.39	0.39
FSHD/10	Thiomethyl-chlorobenzamide	-	0.18	0.18
USHD/18, FSHD/11	M-01	7.65	5.35	13.00
USHD/19	O-sulfate conjugate of dichlorophenyl	0.70	-	0.70
Total identified		63.56	6.54	70.10
USHD/1	Unknown	0.38	-	0.38
USHD/2	Unknown	0.54	-	0.54
USHD/5	Unknown	0.67	-	0.67
USHD/12	Unknown	1.30	-	1.30
USHD/13	Unknown	0.54	-	0.54
USHD/14	Unknown	0.26	-	0.26
USHD/17	Unknown	0.25	-	0.25
USHD/20	Unknown	0.15	-	0.15
FSHD/1	Unknown	-	0.37	0.37
FSHD/2	Unknown	-	0.16	0.16
FSHD/3	Unknown	-	0.25	0.25
FSHD/5	Unknown	-	0.20	0.20
FSHD/6	Unknown	-	0.39	0.39
FSHD/9	Unknown	-	0.11	0.11
FSHD/12	Unknown	-	0.21	0.21
Total characterized		4.09	1.69	5.78
Solids of faeces *				2.15
Urine not analysed (120 - 168 h)				1.67
Faeces not analysed (72 - 168 h)				2.01
Cage wash (0 – 168 h)				9.33
Total				91.04

* Not given in report, calculated from reported data

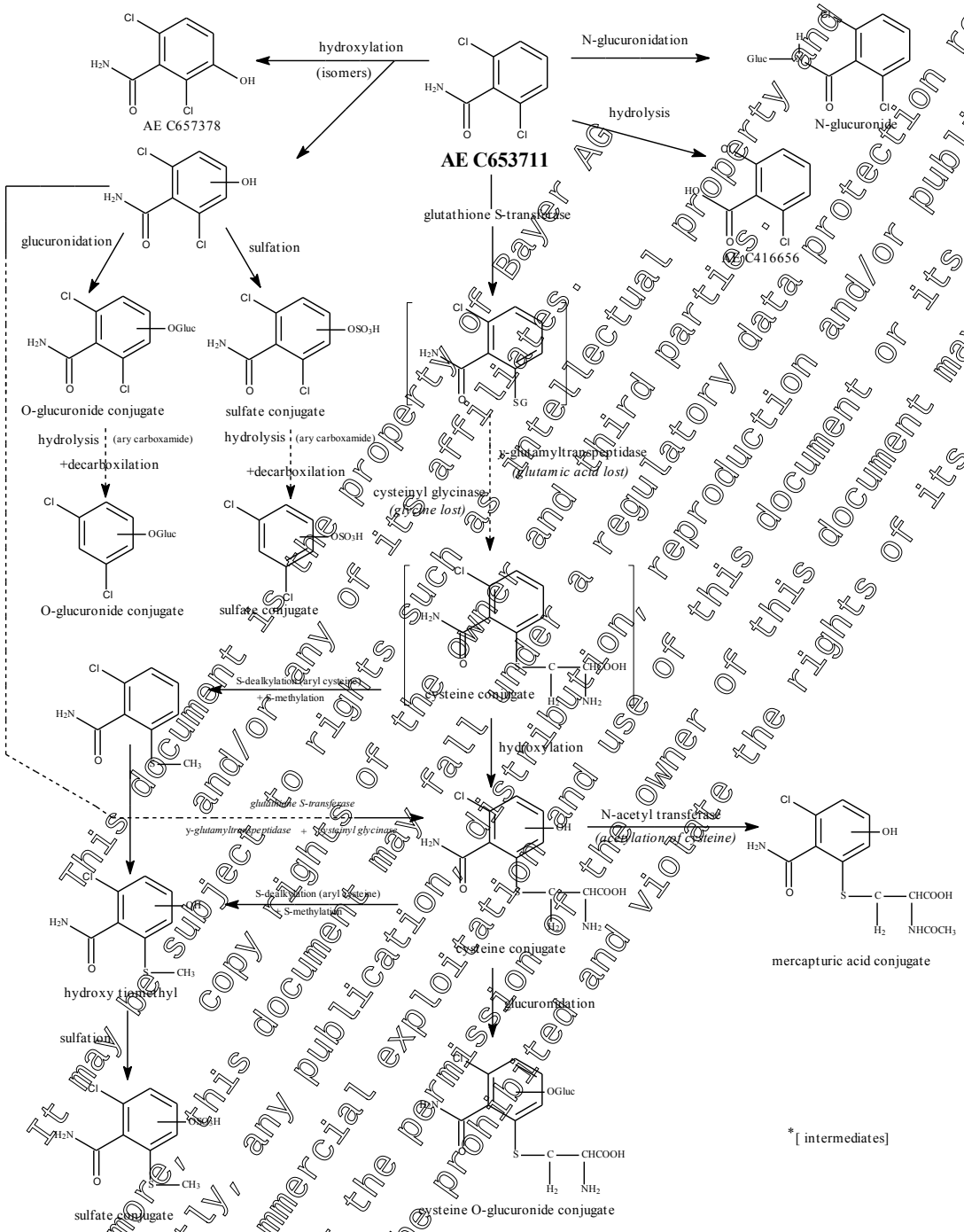
Table 5.1.1- 69: Balance of [phenyl-UL-^{14}C]-M-01 and metabolites excreted after oral administration to female rats (expressed as % dose administered)

Peak ID	Name	Test 2 (female, 150 mg/kg bw)		
		Urine (0 - 120 h)	Faeces (0 - 72 h)	Total
		% of dose administered		
USHD/3	Cysteine and O-glucuronide conjugate of chlorobenzamide	3.78	-	3.78
USHD/4	O-glucuronide conjugate of dichlorobenzamide	2.17	-	2.17
USHD/6	Cysteine conjugate of hydroxy-chlorobenzamide	10.76	-	10.76
USHD/7	AE C416656	0.65	-	0.65
USHD/8	N-glucuronide conjugate of dichlorobenzamide	0.43	-	0.43
USHD/9, FSHD/4	Mercapturic acid conjugate of hydroxy-chlorobenzamide	17.85	0.26	18.11
USHD/10	O-sulfate conjugate of dichlorobenzamide O-sulfate conjugate of thiomethyl-chlorobenzamide	12.45	-	12.45
USHD/11	AE C657378	2.65	-	2.65
FSHD/7	Hydroxy-chlorobenzamide	-	0.14	0.14
USHD/15	O-glucuronide conjugate of dichlorophenyl	0.75	-	0.75
USHD/16	Mercapturic acid conjugate of hydroxy-chlorobenzamide	0.56	-	0.56
FSHD/8	Thiomethyl-hydroxychlorobenzamide	-	0.61	0.61
FSHD/10	Thiomethyl-chlorobenzamide	-	0.09	0.09
USHD/18, FSHD/11	M-01	17.85	6.75	24.60
USHD/19	O-sulfate conjugate of dichlorophenyl	0.81	-	0.81
Total identified		70.71	7.85	78.56
USHD/1	unknown	0.30	-	0.3
USHD/2	unknown	0.46	-	0.46
USHD/5	unknown	1.79	-	1.79
USHD/12	unknown	0.45	-	0.45
USHD/13	unknown	0.09	-	0.09
USHD/14	unknown	1.04	-	1.04
USHD/17	unknown	0.33	-	0.33
FSHD/1	unknown	-	0.53	0.53
FSHD/2	unknown	-	0.15	0.15
FSHD/3	unknown	-	0.15	0.15
FSHD/5	unknown	-	0.19	0.19
FSHD/6	unknown	-	0.11	0.11
FSHD/9	unknown	-	0.11	0.11
FSHD/12	unknown	-	0.53	0.53
Total characterised		4.46	1.77	6.23
Solids of faeces *				1.21
Urine not analysed (120 - 168 h)				1.94
Faeces not analysed (72 - 168 h)				2.23
Cage wash (0 - 168 h)				6.20
Total				96.97

* Not given in report, calculated from reported data

The following metabolic pathway is proposed:

Figure 5.1.1-17: Proposed Metabolic Pathway for [^{14}C]-M-01 (BAM, AE C653711) following a single oral dose at the rate of 150 mg/kg b/w



M-01 (BAM) referred to as AE C653711 and M-04 (3-hydroxy BAM) as AE C657378 in the report

III. Conclusions

The majority of [phenyl-UL-^{14}C]-M-01 administered to rats was eliminated in urine, where the rate of elimination was relatively slow, whilst only low levels were eliminated via the faeces. High levels of oral bioavailability were seen in both male and female rats. Quantification of radioactive residues in tissues showed low residual levels with mean values of 1.17% and 1.21% of dose for male and female rats respectively. The highest concentrations in tissues were seen in skin & fur, liver and kidney, where the mean values ranged between 2.10 and 5.10 µg equivalents/g.

Unchanged M-01 was seen in urine and faecal samples from male and female rats. The major metabolite was USHD/9 which represented ca. 21% dose and 19% dose in male and female urine respectively and was identified to be a mercapturic acid conjugate of hydroxy-chlorobenzamide. Metabolite USHD/9 was obtained from a complex metabolic pathway that included the activities of GSH transferase and peptidase enzymes leading to a cysteinyl conjugate that was subsequently N-acetylated to obtain USHD/9. The biotransformation of M-01 also included the activities of dealkyl-S-cysteine, O-glucuronidase, O-sulfatase enzymes and N-glucuronidase enzymes.

Assessment and conclusion by applicant:
An acceptable study yielding valid conclusions

Data Point:	KCA 5.1.1/15
Report Author:	[redacted]
Report Year:	2003
Report Title:	(Phenyl-U-14C)-AE C653711 (BAM): Single oral low dose A.D.M.E. study in the rat
Report No:	C035245
Document No:	M-218350-01-1
Guideline(s) followed in study:	EU (=EEC): 94/79/EC, 1994; JMAFF 12, Nohsan No. 8147, 200; USEPA (=EPA): OPPTS 870.7485, 1998
Deviations from current test guideline:	None
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

The absorption, distribution, excretion and metabolism of [phenyl-UL-^{14}C]- M-01 (referred to as AE C653711 in the report) was investigated in male and female Sprague Dawley rats following a single oral dose level of 10 mg/kg bw.

[Phenyl-UL-^{14}C]-M-01 was administered orally to two groups of four male or female rats at a low dose of 10 mg/kg bw. Urine and faeces samples were collected for six days until sacrifice, when organs, GIT, skin and carcass were collected.

Mean recoveries were 97% of the administered dose for male rats and 98% for female rats. Oral absorption was 83% of dose for male rats and 86% for females as calculated from the sum of radioactivity in urine, cage washes and tissues.

The routes and rates of elimination were very similar between male and female rats. Excretion of radioactivity was relatively slow, with 96 hours required for close to complete elimination of the total radioactivity in urine. 97 and 96% of the administered dose was excreted in urine, faeces, or cage wash with ≤ 2.2% remaining in the body. At sacrifice (144 hours), rats had excreted 81 to 84% of the dose in urine plus cage washes and 12 to 14% with the faeces. Overall, the quantification of radioactive residues in tissues was low with concentrations below 0.600 µg equivalents/g in all tissues. Highest tissue residues were found in liver and kidney with mean values of 0.439 and 0.566 µg equivalents/g respectively in male rats. A similar distribution pattern was seen in female rats, where the mean levels of radioactivity in liver and kidney were 0.445 and 0.556 µg equivalents/g respectively.

[Phenyl-UL-^{14}C]-M-01 was intensively metabolised with up to 14 radioactive components detected in urine, and 6 in the faecal extracts. Seven metabolites, plus the test item, were identified in urine and faeces. The proposed biotransformation pathway of M-01 in the rat is shown in figure 5.1.1-18.

Unchanged M-01 was identified in urine and faecal samples from male and female rats, totalling 14% of the dose.

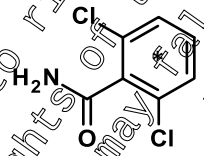
Biotransformations observed included aromatic ring hydroxylation, hydrolysis, decarboxylation, acetylation, and conjugation with glucuronic acid, sulfate, and glutathione. Glutathione conjugates were further metabolised by loss of glycine and glutamic acid to leave cysteine conjugates. The cysteine conjugates were further metabolised by acetylation to form the mercapturic acids or to dealkylated and S-methylated to form S-methyl metabolites. The principal metabolic pathway was formation of a mercapturic acid conjugate of hydroxy-chlorobenzamide present in urine at 25-26% of the dose (USLD/6).

I. Materials and methods

A. Materials

1. Test material: [Phenyl-U-^{14}C]-M-01 (referred to as AE C653711 in the report)

Chemical structure:



* labelling position

Batch no.: SEIc1059

Specific radioactivity: Initial: 2.246 GBq/mmole (60.9 mCi/mmole)
Final: 75 MBq/mmole (10.67 µCi/mg)

Radiochemical purity: 99.15% (HPLC)

2. Vehicle: 0.75% methyl-cellulose in water

3. Test animals:

Species: Rat *(Rattus norvegicus domesticus)*

Strain: Sprague-Dawley CD

Age: 6 to 10 weeks

Weight at dosing: 172 to 195 g (males) and 161 to 171 g (females)

Source: [redacted]

Acclimation period: At least 5 days

Identification: Animals were identified by ear tattoo

Diet:	Certified rodent diet A04C, obtained from UAR, Villemoisson, France The rodent diet was removed approximately 18 hours before administration of the radiolabelled test material and replaced one hour post dosing.
Water:	Filtered and softened water obtained from the municipal supply that was routinely analysed. to ensure that no contaminants were present that could affect the outcome of the study
Housing:	Animals were kept individually in Jencon's Metabowls Mk III metabolism units
Environmental conditions	
Temperature:	22 ± 2°C.
Humidity:	55 ± 15%.
Ventilation:	Average of 10 to 15 air changes per hour
Photoperiod:	12 hour light & 12 hour dark.

4. Preparation of dosing solutions

A stock solution radiolabelled M-01 (SEL-1059) dissolved in acetonitrile was mixed with non-radiolabelled test material (Batch number K001724) to obtain a homogeneous solution. For each test, an adequate portion of the solution was evaporated under a gentle stream of nitrogen and the residue resuspended in 0.75% methyl-cellulose in water.

B. Study design and methods

1. Dose regimen and design of tests

Test no.	Administered single dose of ^{14}C-M-01, route (experiment)	^{14}C-label	Number of rats and sex	Collection of samples during the test and at sacrifice	Duration
1	10 mg/kg bw, oral (single low dose)	phenyl-UL	4 males	expired air, urine, faeces, cage washes, blood, plasma, organs, GIT, skin, carcass	144 h
2	10 mg/kg bw, oral (single low dose)	phenyl-UL	4 females	expired air, urine, faeces, cage washes, blood, plasma, organs, GIT, skin, carcass	144 h

The rats were given a single dose of radiolabelled [phenyl-UL-^{14}C]-M-01 uniformly labelled in the aromatic ring.

[Phenyl-UL-^{14}C]-M-01 was administered orally to groups of four male or female rats at a low dose of 10 mg/kg bw. Expired air was collected for 48 hours post dosing. Urine, cage washes and faeces were collected for six days until sacrifice, when blood, plasma, organs, GIT, skin and carcass were also collected.

2. Dosing

Suspensions of the test compound in 0.75% methyl-cellulose in water were administered orally to each rat. The test suspension was administered by gavage at a rate of 5g dose suspension per kg of rat body weight.

The dose suspension was assayed for test item concentration (by HPLC) and for radioactivity content (by LSC) at three time points (before, mid-way through and after the dosing procedure). Actual dose levels were close to nominal levels:

Test ID	Nominal Dose (mg/kg bw)	Actual Dose (mg/kg bw)
1	10	10.675
2	10	10.688

3. Collection of excreta

After the administration of the radiolabelled test substance, the rats were kept individually in metabowl units, which allowed for separate and quantitative collection of urine, faeces and expired carbon dioxide.

Urine was collected separately for each rat in a cryogenic trap cooled with dry ice at the following intervals after administration of the radiolabelled dose:

6, 24, 48, 72, 96, 120 and 144 hours in tests 1 and 2

Metabowl cages were washed with distilled water every 24 hours and with acetonitrile after the animals had been removed from the units at the end of the in-life phase.

The radioactivity in urine and metabowl rinses was determined by LSC.

Faeces were collected separately for each rat every 24 hours after administration of the radiolabelled dose. Faeces samples were homogenised with approximately twice their volume of water. The radioactivity was determined by combustion followed by LSC.

An airflow of approximately 0.3-0.4 l/min was drawn by electrically powered pumps through each metabolism unit. Exhaled carbon dioxide was trapped in a mixture of ethanolamine in 2-ethoxyethanol (1:2 v/v). The radioactivity was determined by LSC.

4. Expired air

Expired carbon dioxide was collected from 0-6 hours, 6-24 hours and 24-48 hours post dosing. No radioactivity was detected up to 48 hours post dosing and the collection was stopped.

5. Sacrifice

The rats were anaesthetized by injection of Imalgene-500 and sacrificed by exsanguinated.

6. Blood, tissues and organs at sacrifice

At sacrifice, cardiac blood was collected, and plasma prepared by centrifugation. The radioactivity in blood was determined by combustion and in plasma by LSC directly.

The following organs and tissues were collected:

- liver, kidneys, heart, lungs, brain, cardiac blood, spleen, pancreas, muscle, abdominal fat, ovaries (for females), testes (for males), stomach plus contents, intestine plus content, bone and marrow, adrenals, the skin & fur, uterus (for females), eyes, Harderian glands and thyroids

The residual carcass was also retained for analysis.

Fat, testes, bone (plus marrow), uterus, as well as whole tissues such as ovaries, eyes, Harderian glands, thyroids, adrenals were minced by hand with scissors. Other tissues were homogenised using an Ultra-Turrax with small amounts of water when necessary. The radioactivity was determined by combustion followed by LSC

The skin & fur, uterus and carcass samples were solubilised in alcoholic 2M KOH for 24 hours at 50°C. The radioactivity of the solubilised material was determined by LSC.

7. Sample handling and storage

Whenever possible samples were processed as they were collected. Remaining samples and samples awaiting processing were stored at -20°C in the dark until required.

8. Preparation of samples for analysis

Urine and faecal samples contained almost the entire amount of the recovered radioactivity and therefore, the metabolism study was performed with these two categories of samples. Samples were combined to representative pools for each test group.

Pools of urine samples were prepared to represent the following excretion intervals:

0 – 6, 6 – 24, 24 – 48 and 48 – 72 hours for test 1 and 2

Urine samples were concentrated prior to analysis by HPLC.

Pools of faeces samples were prepared to represent the following excretion intervals:

0 - 24 hours for test 1 and 2

Faecal samples were centrifuged for 20 minutes, the aqueous supernatant decanted and the remaining pellet extracted with acetonitrile. Extracts were concentrated prior to analysis by HPLC.

Radioactivity in extracts was determined by LSC and in the remaining solids by combustion followed by LSC.

9. Analytical methods

The chromatographic separation and quantification of the metabolites present in urine and faecal samples was performed using the High Performance Liquid Chromatography (HPLC) with radiodetection and by LC-MS/MS methods.

II. Results and Discussion

A. Recovery

The mean recovery over a six-day period post-administration was found to be 96.55% in male rats and 97.97% in female rats. A summary of the radioactivity as percent of the administered dose found in urine, faeces and in organs and tissues at sacrifice is presented in the table below.

Table 5.1.1- 70: Recovery of radioactivity after administration of [phenyl-UL-^{14}C]-M-01 (BAM) at rate of 10 mg/kg b/w

Percent of administered dose (mean values)

Sample	Test 1 male oral 10 mg/kg bw		Test 2 female oral 10 mg/kg bw	
	Mean	**SD**	**Mean**	**SD**
Urine	66.43	6.10	70.85	5.42
Cage wash	14.39	5.77	13.38	5.04
Faeces	13.53	1.48	12.03	1.75
Carbon dioxide traps	n.d.	n.a.	n.d.	n.a.
Tissues	2.21	0.16	1.71	0.10
Total	**96.55**	**1.30**	**97.97**	**1.64**

SD = standard deviation, n.d. = not detected,

The major route of excretion was *via* the urine for both sexes with a mean of 80.82% of the administered dose in male rats and 84.23% in female rats eliminated (urine plus cage washes) during the 6-day sampling period. The proportion of radioactivity eliminated *via* the faecal route was 13.53% in male rats and 12.03% in female rats.

B. Absorption

The estimated minimum levels of absorption were measured as the total radioactivity in urine, cage washes plus tissues. The results showed that at least 83% of the administered dose was absorbed by male rats and similar results were seen in female rats with 86% of dose absorbed. Therefore, high oral bioavailability was seen in both male and female rats.

C. Distribution

Levels of radioactivity remaining in tissues at 144 hours represented a sum total of 2.21% of the administered dose in male rats and 1.71% in female rats. The distribution of radioactive residues in the rat are summarised in the table below expressed as parent equivalent concentrations in µg/g.

Overall, the quantification of radioactive residues in tissues showed low levels for both male and female rats, with concentrations below 0.600 µg equivalents/g in all tissues. The highest tissue concentrations in male rats were found in liver (mean: 0.439 µg equivalents/g) and kidney (0.566 µg equivalents/g). Similar concentrations were seen in female tissues, where the mean levels of radioactivity in liver and kidney were 0.445 and 0.556 µg equivalents/g, respectively.

Table 5.1.1- 71: Concentration of [phenyl-UL-^{14}C]-M-01 (BAM) residues in rat tissues following a single oral dose at 10 mg/kg bw

µg M-01 equivalents/g tissue

Organs/ Tissues	Test 1 Oral Male 10 mg/kg bw		Test 2 oral female 10 mg/kg bw	
	Mean	SD	Mean	SD
Cardiac blood	0.057	0.014	0.043	0.004
Intestine & contents	0.103	0.022	0.076	0.012
Harder's Gland	0.350	0.030	0.329	0.021
Residual Carcass	0.115	0.018	0.113	0.015
Skin & Fur	0.350	0.022	0.321	0.071
Cardiac Plasma	0.050	0.014	0.034	0.004
Eyes	0.058	0.012	0.039	0.008
Brain	0.073	0.013	0.049	0.007
Fat	0.042	0.007	0.029	0.006
Heart	0.161	0.027	0.149	0.008
Lungs	0.099	0.020	0.085	0.015
Spleen	0.075	0.017	0.054	0.007
Liver	0.439	0.041	0.445	0.081
Kidneys	0.566	0.066	0.556	0.057
Stomach & contents	0.054	0.009	0.057	0.031
Thyroids	0.154	0.049	n.a.	n.a.
Ovaries	n.a.	n.a.	0.054	0.007
Testes	0.074	0.015	n.a.	n.a.
Pancreas	0.098	0.019	0.083	0.011
Uterus	n.a.	n.a.	0.041	0.005
Adrenal	0.262	0.089	0.274	0.028
Muscle	0.098	0.011	0.088	0.005
Bone & Marrow	0.038	0.010	0.030	0.006

SD = standard deviation, n.a. = not applicable

D. Excretion

The rate of elimination was slow with 96 hours being necessary for the excretion of *ca*. 95% of the total radioactivity excreted in urine. The routes and rates of elimination were very similar between male and female rats. These data are presented in the table below.

Table 5.1.1- 72: Elimination of radioactivity after administration of [phenyl-UL-^{14}C]-M-01 (BAM) at rate of 10 mg/kg b/w

Percent of administered dose (mean values)

Sample	Time [h post admin.]	Test 1 Male Oral 10 mg/kg bw		Test 2 female oral 10 mg/kg bw	
		Mean	SD	Mean	SD
Urine	0-6	3.44	0.467	3.56	1.272
	6-24	36.03	3.393	38.72	4.081
	24 -48	53.40	4.036	59.48	5.765
	48 -72	60.61	5.146	66.64	5.332
	72-96	64.17	5.574	69.22	5.581
	96-120	65.69	5.953	70.35	5.438
	120-144	66.43	6.103	70.85	5.416
Faeces	0-24	6.71	1.641	5.82	1.21
	24 -48	10.81	1.599	9.48	1.44
	48 -72	12.34	1.587	10.99	1.78
	72-96	13.04	1.526	11.60	1.719
	96-120	13.36	1.501	11.85	1.747
	120-144	13.52	1.481	12.03	1.749
Cage wash	0-144	14.39	5.773	13.38	5.042
Total eliminated		**94.34**	**1.393**	**96.26**	**1.734**

SD = standard deviation

E. Metabolism

The unchanged test item, M-01, was one of the major components in urine and faeces reaching a sum total of 14% in males and females (10.3% in male urine, 11.2% in female urine, 3.6% in male faeces and 3.3% in female faeces).

Three different metabolic pathways were postulated for the biotransformation of M-01. One of them was the hydrolysis of the test product leading to the dichlorobenzoic acid (AE C416656) which accounted for ≤ 0.05% dose in rat urine.

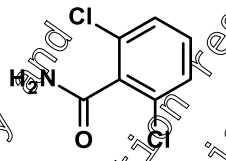
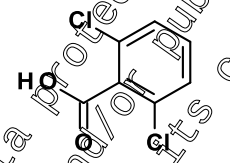
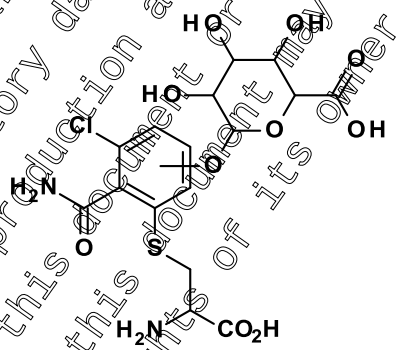
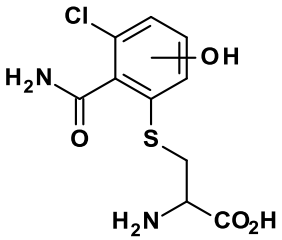
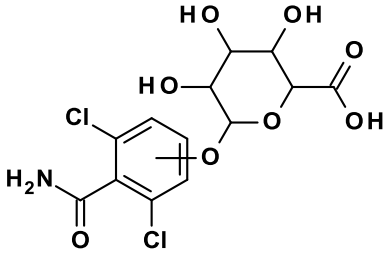
The principal metabolic pathway was the formation of a mercapturic acid conjugate of hydroxy-chlorobenzamide USLD/6. USLD/6 was the major metabolite eliminated in the urine accounting for *ca.* 26% dose in male urine and 25% in female urine. There were two potential pathways that led to the mercapturic acid conjugate. The first was aromatic dehalogenation of M-01 followed by the action of glutathione S-transferase resulting in a glutathione conjugate intermediate. The GS-intermediate was further biotransformed by the loss of glutamic acid and glycine leading to a cysteine conjugate that was subsequently hydroxylated in the aromatic ring, resulting in USLD/4. The metabolite USLD/4 was the cysteine conjugate of hydroxy-chlorobenzamide that was subsequently N-acetylated leading to its derivative mercapturic acid conjugate.

The second proposed metabolic pathway leading to USLD/6 was the aromatic hydroxylation on the test item leading to USLD/4 followed by dehalogenation, the action of GSH enzymes and the loss of glutamic acid and glycine. The metabolic pathway provides further details of the two biotransformation pathways leading to the mercapturic acid conjugate. The hydroxy metabolite of the test item was named (intermediate 1, identified in M-218352-01-1) was subsequently metabolised by the action of glucuronidase and sulfatase enzymes leading to O-glucuronide (USLD 3a) and O-sulphate conjugates (USLD 8a).

The metabolite USLD/4 accounted for 1.87% in male rats and 9.10% in female rats, USHD/4 was further metabolised by the action of glucuronide enzymes leading to USLD/2 the O-glucuronide conjugate of USLD/4. The metabolite USLD/3 accounted for a total of 6.35% in male urine and 6.57% in female urine. A different biotransformation of USHD/4 was obtained by the action of S-dealkylation enzymes (aryl cysteine lost) leading to thiomethyl hydroxy-chlorobenzamide (intermediate 3, identified in M-218352-01-1) which was subsequently metabolised by the action of sulfatase enzymes leading to O-sulphate conjugate of thiomethyl-chlorobenzamide (USLD/8b).

The following table below presents a summary of the identified metabolites. The levels of M-01 and its metabolites quantified in urine and faeces are provided in table 5.1.1-74 and table 5.1.1-75 for male and female rats, respectively.

Table 5.1.1- 73: Identified metabolites

Report name (peak ID in the profiles)	Chemical name (IUPAC)	Chemical Structure
Test item M-01 (M-01, BCS-AA65784, BAM) peak ID in the profiles: USLD/13 and FSLD/11	2,6-dichlorobenzamide	
AE C416656 peak ID in the profiles: USLD/5B	2,6-dichlorobenzoic acid	
USLD/2 Cysteine and O-glucuronide conjugate of chlorobenzamide	Depending on the position of the glucuronide conjugation IUPAC: 6-[4-(2-amino-2-carboxy-ethyl)sulfanyl-3-carbamoyl-2-chloro-phenoxy]-3,4,5-trihydroxy-tetrahydropyran-2-carboxylic acid Or 6-[3-(2-amino-2-carboxy-ethyl)sulfanyl-4-carbamoyl-5-chloro-phenoxy]-3,4,5-trihydroxy-tetrahydropyran-2-carboxylic acid Or 6-[2-(2-amino-2-carboxy-ethyl)sulfanyl-3-carbamoyl-4-chloro-phenoxy]-3,4,5-trihydroxy-tetrahydropyran-2-carboxylic acid	
USLD/4 Cysteine conjugate of hydroxy-chlorobenzamide	Depending on the position of the hydroxylation IUPAC: 2-amino-3-(2-carbamoyl-3-chloro-5-hydroxy-phenyl)sulfanyl-propanoic acid Or 2-amino-3-(2-carbamoyl-3-chloro-6-hydroxy-phenyl)sulfanyl-propanoic acid Or 2-amino-3-(2-carbamoyl-3-chloro-4-hydroxy-phenyl)sulfanyl-propanoic acid	
USLD/5a O-Glucuronide conjugate of dichlorobenzamide	Depending on the position of the glucuronide conjugation IUPAC: 6-(4-carbamoyl-3,5-dichloro-phenoxy)-3,4,5-trihydroxy-tetrahydropyran-2-carboxylic acid Or 6-(3-carbamoyl-2,4-dichloro-phenoxy)-3,4,5-trihydroxy-tetrahydropyran-2-carboxylic acid	

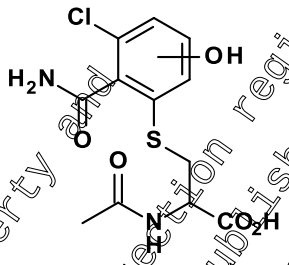
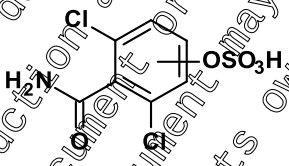
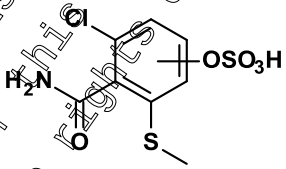
Report name (peak ID in the profiles)	Chemical name (IUPAC)	Chemical Structure
USLD/6 Mercapturic acid conjugate of hydroxy-chlorobenzamide	Depending on the position of the hydroxylation IUPAC: 2-acetamido-3-(2-carbamoyl-3-chloro-5-hydroxy-phenyl)sulfanyl-propanoic acid Or 2-acetamido-3-(2-carbamoyl-3-chloro-6-hydroxy-phenyl)sulfanyl-propanoic acid Or 2-acetamido-3-(2-carbamoyl-3-chloro-4-hydroxy-phenyl)sulfanyl-propanoic acid	
USLD/8 a O-Sulfate conjugate of dichlorobenzamide	Depending on the position of the O-sulfate conjugate IUPAC: (4-carbamoyl-3,5-dichloro-phenyl) hydrogen sulfate Or (3-carbamoyl-2,4-dichloro-phenyl) hydrogen sulfate	
USLD/8 b O-Sulfate conjugate of thiomethyl-chlorobenzamide	Depending on the position of the O-sulfate conjugate IUPAC: (4-carbamoyl-3-chloro-2-methylsulfanyl-phenyl) hydrogen sulfate Or (3-carbamoyl-2-chloro-4-methylsulfanyl-phenyl) hydrogen sulfate Or (3-carbamoyl-4-chloro-2-methylsulfanyl-phenyl) hydrogen sulfate	

Table 5.1.1- 74: Balance of [phenyl-UL-^{14}C]-M-01 (BAM) and metabolites excreted after oral administration to male rats (expressed as % dose administered)

Peak ID	Name	Test 1 (male, 10 mg/kg bw)		
		Urine (0 - 72 h)	Faeces (0 - 24 h)	Total
		% of dose administered		
USLD/2	Cysteine and O-glucuronide conjugate of chlorobenzamide	6.38	-	6.35
USLD/4	Cysteine conjugate of hydroxy-chlorobenzamide	1.87	-	1.87
USLD/5	O-Glucuronide conjugate of dichlorobenzamide AE C416656	0.02	-	0.02
USLD/6	Mercapturic acid conjugate of hydroxy-chlorobenzamide	26.24	-	26.24
USLD/8	O-sulfate conjugate of dichlorobenzamide O-sulfate conjugate of thiomethyl-chlorobenzamide	6.21	-	6.21
USLD/13, FSLD/6	M-01	10.33	3.61	13.94
Total identified		51.02	3.61	54.63
USLD/1	Unknown	0.34	-	0.34
USLD/3	Unknown	4.70	-	4.70
USLD/7	Unknown	1.06	-	1.06
USLD/9	Unknown	1.97	-	1.97
USLD/10	Unknown	0.11	-	0.11
USLD/12	Unknown	0.12	-	0.12
USLD/14	Unknown	1.08	-	1.08
FSLD/1	Unknown	-	0.40	0.40
FSLD/2	Unknown	-	0.17	0.17
FSLD/3	Unknown	-	0.07	0.07
Total characterized		9.38	0.64	10.02
Solids of faeces *				2.67
Urine not analysed (72 - 144 h)				5.82
Faeces not analysed (24 - 144 h)				6.81
Cage wash (0 – 144 h)				14.39
Total				94.34

* Not given in report, calculated from reported data

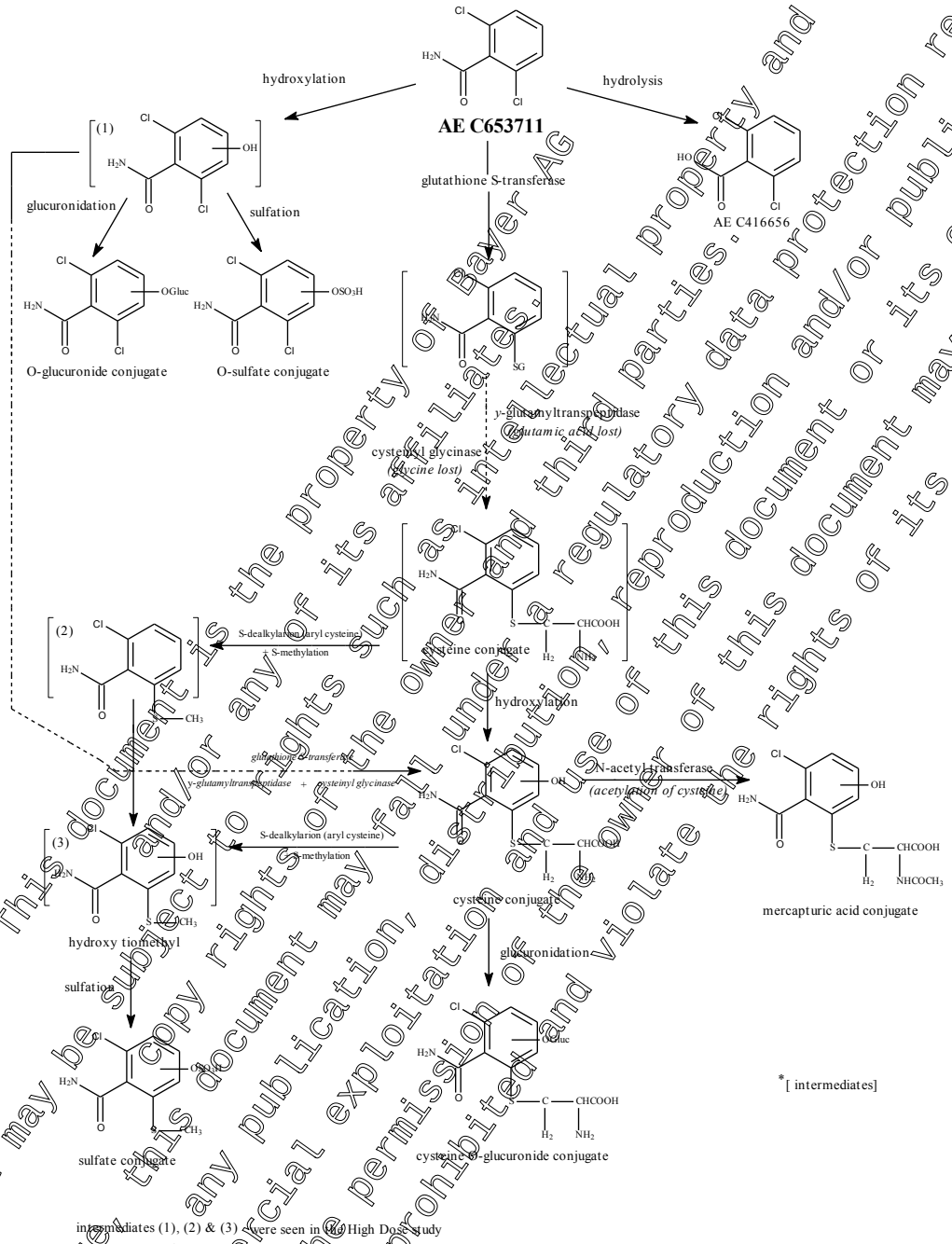
Table 5.1.1- 75: Balance of [phenyl-UL-^{14}C]-M-01 (BAM) and metabolites excreted after oral administration to female rats (expressed as % dose administered)

Peak ID	Name	Test 2 (female, 10 mg/kg bw)		
		Urine (0 - 72 h)	Faeces (0 - 24 h)	Total
		% of dose administered		
USLD/2	Cysteine and O-glucuronide conjugate of chlorobenzamide	6.57	-	6.57
USLD/4	Cysteine conjugate of hydroxy-chlorobenzamide	9.10	-	9.10
USLD/5	O-Glucuronide conjugate of dichlorobenzamide AE C416656	0.05	-	0.05
USLD/6	Mercapturic acid conjugate of hydroxy-chlorobenzamide	25.37	-	25.37
USLD/8	O-sulfate conjugate of dichlorobenzamide O-sulfate conjugate of thiomethyl-chlorobenzamide	6.16	-	6.16
USLD/13, FSLD/6	M-01	11.23	3.30	14.53
Total identified		58.48	3.30	61.78
USLD/1	Unknown	0.65	-	0.65
USLD/3	Unknown	2.21	-	2.21
USLD/7	Unknown	1.24	-	1.24
USLD/9	Unknown	1.72	-	1.72
USLD/10	Unknown	0.31	-	0.31
USLD/11	Unknown	0.01	-	0.01
USLD/12	Unknown	0.47	-	0.47
USLD/14	Unknown	1.19	-	1.19
FSLD/1	Unknown	-	0.04	0.04
FSLD/3	Unknown	-	0.11	0.11
FSLD/4	Unknown	-	0.07	0.07
FSLD/5	Unknown	-	0.15	0.15
Total characterized		7.80	0.37	8.17
Solids of faeces *				2.52
Urine not analysed (72 - 144 h)				4.21
Faeces not analysed (24 - 144 h)				6.20
Cage wash (0 - 144 h)				13.38
Total				96.26

* Not given in report, calculated from reported data

The following metabolic pathway is proposed:

Figure 5.1.1-18: Proposed Metabolic Pathway for [^{14}C]-M-01 (BAM, AE C653711) following a single oral dose at the rate of 10 mg/kg b/w



III. Conclusions

The majority of [phenyl-UL-^{14}C]-M-01 administered to rats was eliminated in urine, where the rate of elimination was relatively slow, whilst only low levels were eliminated via the faeces. High levels of oral bioavailability were seen in both male and female rats. Quantification of radioactive residues in tissues showed low residual levels with mean values of 2.21% and 1.71% of dose for male and female rats respectively. Overall, the quantification of radioactive residues in tissues showed low concentrations for both male and female rats, where the mean values were all below 0.600 µg equivalents/g. Unchanged M-01 was seen in urine and faecal samples from male and female rats. The major metabolite was USHD/6 which represented ca. 26% dose and 25% dose in male and female urine respectively and was identified to be a mercapturic acid conjugate of hydroxy-chlorobenzamide. The mercapturic acid conjugate was obtained from a complex metabolic pathway that included the activities of GSH transferase and peptidase enzymes leading to a cysteinyl conjugate that was subsequently N-acetylated to obtain USHD/6. The biotransformation of M-01 also included the activities of dealkyl-S-cysteine, O-glucuronidase and O-sulfatase enzymes.

Assessment and conclusion by applicant:
An acceptable study yielding valid conclusions

Data Point:	KCA 5.1.1/16
Report Author:	[redacted]
Report Year:	2003
Report Title:	Repeat oral low dose A.D.M.E. study in the rat Code: (phenyl-U-14C)-AE C653711
Report No:	C035920
Document No:	M-219491-01-1
Guideline(s) followed in study:	EU (=EEC): 94/79/EC, 1994, JMAF 12, Nohsan No. 8147, 200; USEPA (=EPA): OPPTS 870.7485, 1998
Deviations from current test guideline:	None
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

The absorption, distribution, excretion and metabolism of [phenyl-UL-^{14}C]-M-01 (BAM, referred to as AE C653711 in the report) was investigated in male and female Sprague Dawley rats following repeated oral dosing at a nominal dose level of 10 mg/kg bw.

[Phenyl-UL-^{14}C]-M-01 was administered orally to two groups of five male or female rats at a low dose of 10 mg/kg bw/day for 14 days. Urine and faeces samples were collected throughout the dosing period and for a further six days after the final dose until sacrifice, when organs, GIT, skin and carcass were collected.

Mean recoveries were 97% of the administered dose for male rats and 99% for female rats. Oral absorption after multiple dosing was a minimum of 78% in male rats and 83% for females as calculated from the sum of radioactivity in urine, cage washes and tissues.

The routes and rates of elimination were similar between male and female rats. The main route of elimination was via urine, with 76.67% of the administered dose being eliminated via this route in males (53.42% plus 23.25% in the cage-wash). Similarly, in females, 82.41% of the administered dose was eliminated via the urine (68.88% plus 13.53% in the cage-wash).

Overall, the multiple dosing did not have any significant impact in the absorption, distribution, metabolism and elimination compared to results after single oral dosing. The administered dose was excreted in urine, faeces, or cage wash with ≤ 1.1% remaining in the body. At sacrifice (144 hours after the final dose), rats had excreted 77 to 82% of the dose with the urine plus cage washes and 16 to 19% with the faeces. The highest tissue residues were seen in skin & fur with mean values of 3.169 and 2.846 µg equivalents/g for male and female rats respectively. Liver and kidney contained concentrations of 1.672 and 2.713 µg equivalents/g for male rats and 0.829 and 1.075 µg equivalents/g for females. The adrenals contained mean concentrations of 1.379 and 0.410 µg equivalents/g for male and female rats respectively. All other tissues contained mean residues below 1.000 µg equivalents/g for both male and female rats. Overall, the residue concentration was higher in male rats compared to female rats for all tissues analysed. The radioactive residues in ovaries and uterus were also seen to be lower than in testes.

[Phenyl-UL-^{14}C]-M-01 was intensively metabolised with up to 19 radioactive components detected in urine, and 12 in the faecal extracts. Nine metabolites including 3 isomers, plus the test item, were identified in urine and faeces. The proposed biotransformation pathway of M-01 in the rat is shown in Figure 5.1.1-1.

Unchanged M-01 was identified in urine and faecal samples from male and female rats at a total of *ca.* 20% of the dose.

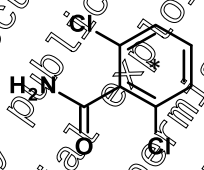
Biotransformations observed included aromatic ring hydroxylation, hydrolysis, decarboxylation, acetylation, and conjugation with glucuronic acid, sulphate and glutathione. Glutathione conjugates were further metabolised by loss of glycine and glutamic acid to leave cysteine conjugates. The cysteine conjugates were further metabolised by acetylation to form the mercapturic acids or to dealkylated and S-methylated to form S-methyl metabolites. The principal metabolic pathway was formation of a mercapturic acid conjugate of hydroxy-chlorobenzamide present in urine at 15 to 16% of the dose (URLD/9).

I. Materials and methods

A. Materials

1. Test material: [Phenyl-UL-^{14}C]-M-01 (referred to as AE C653711 in the report)

Chemical structure:



* labelling position

Batch no.: SEL/1059

Specific radioactivity: Initial: 2.246 GBq/mmole (60.9 mCi/mmole)
Final: 5.0264 to 5.3975 µCi/mg

Radiochemical purity: ≥ 98% (HPLC)

2. Vehicle: 0.75% methyl-cellulose in water

3. Test animals:

Species:	Rat *(Rattus norvegicus domesticus)*
Strain:	Sprague-Dawley CD
Age:	6 to 10 weeks
Weight at dosing:	First treatment: 172 to 177 g (males) and 165 to 173 g (females) Last treatment: 262 to 289 g (males) and 197 to 211 g (females)
Source:	[redacted]
Acclimation period:	At least 5 days
Identification:	Animals were identified by ear tattoo
Diet:	Certified rodent diet A04C, obtained from UAR, Villemoisson, France The rodent diet was removed approximately 18 hours before administration of the radiolabelled test material and replaced one hour post dosing.
Water:	Filtered and softened water obtained from the municipal supply that was routinely analysed to ensure that no contaminants were present that could affect the outcome of the study
Housing:	Animals were kept individually in Jencon's Metabowls Mk III metabolism units
Environmental conditions	
Temperature:	22 ± 2°C.
Humidity:	55 ± 15%.
Ventilation:	Average of 10 to 15 air changes per hour
Photoperiod:	12 hour light & 12 hour dark.

4. Preparation of dosing solutions

A stock solution radiolabelled M-01 (SEL-1059) dissolved in acetonitrile was mixed with non-radiolabelled test material (Batch number R001724) to obtain a homogeneous solution. For each test, an adequate portion of the solution was evaporated under a gentle stream of nitrogen and the residue resuspended in 0.75% methyl-cellulose in water.

B. Study design and methods

1. Dose regimen and design of tests

Test no.	Administered single dose of ^{14}C-M-01, route (experiment)	^{14}C-label	Number of rats and sex	Collection of samples during the test and at sacrifice	Duration
1	10 mg/kg bw/d, oral (repeat low dose for 14 days)	phenyl-UL	5 males	urine, faeces, cage washes, blood, plasma, organs, GIT, skin, carcass	13 days plus 144 hours post final dose (19 days in total)
2	10 mg/kg bw/d, oral (repeat low dose for 14 days)	phenyl-UL	5 females	urine, faeces, cage washes, blood, plasma, organs, GIT, skin, carcass	13 days plus 144 hours post final dose (19 days in total)

The rats were given fourteen daily doses of radiolabelled [phenyl-UL-^{14}C]-M-01 uniformly labelled in the aromatic ring.

[Phenyl-UL-^{14}C]-M-01 was administered orally to groups of five male or female rats at a low dose of 10 mg/kg bw each day. Urine, cage washes and faeces were collected throughout the dosing period and then until sacrifice, which was six days after the final dose, when blood, plasma, organs, GIT, skin and carcass were also collected.

2. Dosing

Suspensions of the test compound in 0.75% methyl-cellulose in water were administered orally to each rat. The test suspension was administered by gavage at a rate of 5g dose suspension per kg of rat body weight.

The dose suspension was assayed for test item concentration (by HPLC) and for radioactivity content (by LSC) at three time points (before, mid-way through and after each dosing procedure). Actual dose levels were close to nominal levels:

Test ID	Nominal Dose (mg/kg bw/d)	Actual Dose (mg/kg bw/d) *
1	10	9.278
2	10	9.293

* Average of each dose, n =65

3. Collection of excreta

After the administration of the radiolabelled test substance, the rats were kept individually in metabowl units which allowed for separate and quantitative collection of urine and faeces.

Urine was collected separately for each rat in a cryogenic trap cooled with dry ice at the following intervals after administration of the radiolabelled dose:

- 0-6 hours, 6-24 hours (pooled to form Day 1 prior to analysis) and at twenty-four hour intervals following dosing up to Day 19.

Metabowl cages were washed with distilled water every 24 hours and with acetonitrile after the animals had been removed from the units at the end of the in-life phase.

The radioactivity in urine and metabowl rinses was determined by LSC.

Faeces were collected separately for each rat every 24 hours following dosing up to Day 19. Faeces samples were homogenised with approximately twice their volume of water. The radioactivity was determined by combustion followed by LSC.

4. Expired air

The collection of expired carbon dioxide and other volatiles was not undertaken in this study as it had been established in the single oral low dose study (M-248350-01-1) that quantitative mass balances were obtained without collection of expired air.

5. Sacrifice

The rats were anaesthetized by injection of Inalgene-500 and sacrificed by exsanguinated 144 hours after the last dose.

6. Blood, tissues and organs at sacrifice

At sacrifice, cardiac blood was collected, and plasma prepared by centrifugation. The radioactivity in blood was determined by combustion and in plasma by LSC directly.

The following organs and tissues were collected:

- liver, kidneys, heart, lungs, brain, cardiac blood, spleen, pancreas, muscle, abdominal fat, ovaries (for females), testes (for males), stomach plus contents, intestine plus content, bone and marrow, adrenals, the skin & fur, uterus (for females), eyes, Harderian glands and thyroids

The residual carcass was also retained for analysis.

Fat was minced by hand with scissors and solubilised in soluene/iso-propanol 50:50 (v/v). The harderian glands, ovaries, eyes, thyroids, adrenals, uterus and pancreas were solubilised in soluene. The skin & fur, intestinal tract & contents, stomach & contents, muscle and carcass samples were solubilised in alcoholic 2M KOH for 24 hours at 50°C. The radioactivity of the solubilised material was determined by LSC.

Bone (plus marrow) was minced by hand with scissors. Other tissues such as liver, kidney, brain, testes, heart, lungs and spleen were homogenised using an Ultra-Turrax with small amounts of water when necessary. The radioactivity was determined by combustion followed by LSC.

7. Sample handling and storage

Whenever possible samples were processed as they were collected. Remaining samples and samples awaiting processing were stored at -20°C in the dark until required.

8. Preparation of samples for analysis

Urine and faecal samples contained almost the entire amount of the recovered radioactivity and therefore, the metabolism study was performed with these two categories of samples. Samples were combined to representative pools for each test group.

Representative urine and faecal samples collected after single dosing (Day 1) and multiple dosing (Day 14) were selected to compare the chromatographic profiles of excreta samples before and after multiple dosing. Urine and faecal samples from Day 14 were used for LC-MS analysis.

Urine samples were concentrated prior to analysis by HPLC.

Faecal samples were centrifuged for 20 minutes, the aqueous supernatant decanted and the remaining pellet extracted twice with acetonitrile. All three extracts were pooled and concentrated prior to analysis by HPLC. Radioactivity in extracts was determined by LSC.

9. Analytical methods

The chromatographic separation and quantification of the metabolites present in urine and faecal samples was performed using the High Performance Liquid Chromatography (HPLC) with radio-detection and by LC-MS/MS methods.

II. Results and Discussion

A. Recovery

Male and female rats received repeated daily oral doses by gavage for a 14-day period with radiolabelled M-01 at a nominal dose rate of 10 mg/kg body weight. The mean recovery over the 14-day dosing period followed by a 6-day period post-dosing was found to be 96.53% in male rats and 98.63% in female rats. A summary of the radioactivity as percent of the administered dose found in urine and faeces 24 hours after the last dose and in urine, faeces, organs and tissues at sacrifice, 6 days after the last dose, is presented in table 5.1.1-76.

Table 5.1.1- 76: Recovery of radioactivity after repeated administration of [phenyl-UL-^{14}C]-M-01 (BAM) at rate of 10 mg/kg b/w

Percent of administered dose (mean values)

Timing	Sample	Test 1 male oral, repeat 10 mg/kg bw/d		Test 2 female oral, repeat 10 mg/kg bw/	
		Mean	SD	Mean	SD
Day 14 (24 hours after last dose	Urine	47.45	6.03	64.08	11.14
	Faeces	16.95	2.72	14.96	4.38
	Cage wash	21.26	4.80	12.45	5.85
	Sub total	***85.67***	***0.83***	***91.50***	***2.96***
Day 19 (Sacrifice, 144 hours after last dose)	Urine	53.42	7.03	68.88	11.59
	Faeces	18.80	2.76	16.24	4.87
	Cage wash	23.25	4.85	13.53	6.01
	Tissues	1.07	0.17	0.59	0.10
	Total	***96.53***	***1.34***	***98.63***	***3.25***

SD = standard deviation

The major route of excretion was *via* the urine for both sexes with a mean of 76.67% of the administered dose in male rats and 82.41% in female rats eliminated (urine plus cage washes) during the study. The proportion of radioactivity eliminated *via* the faecal route was 18.80% in male rats and 16.24% in female rats.

B. Absorption

The estimated minimum levels of absorption were measured as the total radioactivity in urine, cage washes plus tissues. The minimum amount of dose absorbed after multiple dosing was calculated to be *ca.* 77.7% in males and *ca.* 83.0% in females. Therefore, high oral bioavailability was seen in both male and female rats (see table 5.1.1-76).

C. Distribution

Levels of radioactivity remaining in tissues at 144 hours represented a sum total of 1.07% of the administered dose in male rats and 0.59% in female rats. The distribution of radioactive residues in the rat are summarised in table 5.1.1-77, expressed as parent equivalent concentrations in µg/g.

The highest tissue residues were seen in skin & fur with mean values of 3.169 and 2.846 µg equivalents/g for male and female rats respectively. Liver and kidney presented mean values of 1.672 and 2.713 µg equivalents/g for male rats. The radioactive residues found in liver and kidney of female rats presented mean values of 0.829 and 1.075 µg equivalents/g. The adrenals presented mean values of 1.379 and 0.410 µg equivalents/g for male and female rats respectively. All other tissues presented mean values that were below 1.000 µg equivalents/g for both male and female rats. Overall, the residue concentration was higher in male rats compared to female rats for all tissues analysed. The radioactive residues in ovaries and uterus were also seen to be lower than in testes.

Table 5.1.1- 77: Concentration of [phenyl-UL-^{14}C]-M-01 (BAM) residues in rat tissues following repeated oral dosing at 10 mg/kg bw/d

µg M-01 equivalents/g tissue

Organs/ Tissues	Test 1 Oral Male 10 mg/kg bw/d		Test 2 oral female 10 mg/kg bw/d	
	Mean	SD	Mean	SD
Adrenal	1.379	0.311	0.410	0.062
Bone & Marrow	0.334	0.067	0.100	0.019
Brain	0.578	0.147	0.107	0.021
Carcass	0.466	0.099	0.182	0.012
Cardiac blood	0.588	0.141	0.273	0.065
Cardiac Plasma	0.497	0.133	0.093	0.020
Eyes	0.482	0.067	0.087	0.013
Fat	0.267	0.043	0.077	0.009
Harder's Gland	0.901	0.139	0.409	0.034
Heart	0.659	0.139	0.230	0.019
Intestine & contents	0.357	0.042	0.122	0.010
Kidneys	2.713	0.730	1.075	0.140
Liver	1.672	0.934	0.829	0.088
Lungs	0.656	0.103	0.199	0.026
Muscle	0.500	0.069	0.184	0.018
Ovaries	n.a.	n.a.	0.231	0.043
Pancreas	0.493	0.113	0.116	0.049
Skin & Fur	3.169	0.673	2.846	0.738
Spleen	0.674	0.160	0.177	0.026
Stomach & contents	0.583	0.135	0.270	0.047
Testes	0.566	0.085	n.a.	n.a.
Thyroids	0.738	0.183	0.288	0.172
Uterus	n.a.	n.a.	0.111	0.060

SD = standard deviation, n.a. = not applicable

D. Excretion

The routes and rates of elimination were similar between male and female rats. These data are presented in table 5.1.1-78.

Six days after repeated 14 day dosing a total of 53.42% of the administered dose in male rats and 68.88% in female rats was excreted in the urine. The majority of the radioactivity eliminated in urine was excreted within the first 96 hours post dosing, with a total of 52.60% dose in males and 68.55% in females.

The total elimination of radioactivity *via* the faeces was 18.80% of the dose in male rats and 16.24% in female rats. The majority of the radioactivity was excreted within the first 72 hours post dosing (Day 16) where the cumulative values were already 18.28% in male rats and 16.07% in female rats.

Table 5.1.1- 78: Elimination of radioactivity after repeated administration of [phenyl-UL-^{14}C]-M-01 (BAM) at rate of 10 mg/kg b/w

Percent of administered dose (mean values)

Sample	Time [days]	Test 1 male oral 10 mg/kg bw		Test 2 female oral 10 mg/kg bw	
		Mean	SD	Mean	SD
Urine	Day 1	29.22	5.34	33.05	11.72
	Day 2	31.52	4.10	39.25	10.44
	Day 3	34.01	3.89	44.24	7.91
	Day 4	37.39	3.99	46.97	12.35
	Day 5	37.96	4.72	50.70	13.59
	Day 6	39.06	5.04	54.21	12.06
	Day 7	41.60	4.77	56.92	10.80
	Day 8	43.08	4.64	58.18	10.17
	Day 9	43.49	5.25	59.85	11.92
	Day 10	44.19	5.45	61.36	11.30
	Day 11	46.08	5.80	62.67	11.55
	Day 12	46.94	5.51	62.98	11.16
	Day 13	47.15	6.07	63.20	11.41
	Day 14	47.45	6.03	64.08	11.14
	Day 15	50.25	6.47	66.67	11.43
	Day 16	51.65	6.73	67.83	11.54
	Day 17	52.60	6.87	68.55	11.62
	Day 18	53.12	6.96	68.77	11.59
	Day 19	53.42	7.03	68.88	11.59

Sample	Time [days]	Test 1 male oral 10 mg/kg bw		Test 2 female oral 10 mg/kg bw	
		Mean	SD	Mean	SD
Faeces	Day 1	9.64	0.99	10.24	3.37
	Day 2	10.84	1.11	9.86	3.02
	Day 3	11.77	1.04	9.86	2.13
	Day 4	12.96	1.27	13.79	7.27
	Day 5	13.81	1.09	14.26	6.73
	Day 6	14.36	1.26	13.87	5.70
	Day 7	14.64	1.47	13.58	4.98
	Day 8	15.81	2.60	13.96	4.83
	Day 9	16.46	2.97	14.22	4.68
	Day 10	16.61	2.63	14.41	4.36
	Day 11	16.88	3.10	14.71	4.78
	Day 12	16.82	2.84	14.70	4.40
	Day 13	16.77	2.55	14.88	4.13
	Day 14	16.95	2.72	14.96	4.38
	Day 15	17.80	2.67	15.76	4.76
	Day 16	18.28	2.74	16.07	4.85
	Day 17	18.55	2.75	16.18	4.86
	Day 18	18.70	2.76	16.22	4.87
	Day 19	18.80	2.76	16.24	4.87
Cage wash	Day 19	23.25	4.85	13.53	
Total eliminated		**95.47**		**98.65**	

SD = standard deviation

E. Metabolism

The unchanged test item, M-01 (BAM, AE C653711), was one of the major components in urine and faeces reaching a sum total of 20% in males and 19% in females (9.5% in male urine, 11.7% in female urine, 10.4% in male faeces and 7.7% in female faeces).

The principal metabolic pathway was the formation of a mercapturic acid conjugate of hydroxy-chlorobenzamide URLD/9. URLD/9 was the major metabolite eliminated in the urine accounting for *ca.* 15% dose in male urine and 16% in female urine. There were two potential pathways that led to the mercapturic acid conjugate. The first was aromatic dehalogenation of M-01 followed by the action of glutathione S-transferase resulting in a glutathione conjugate intermediate. The GS-intermediate was further biotransformed by the loss of glutamic acid and glycine leading to a cysteine conjugate (intermediate 5) that was subsequently hydroxylated in the aromatic ring, resulting in URLD/6. The metabolite URLD/6 was the cysteine conjugate of hydroxy-chlorobenzamide that was subsequently N-acetylated leading to its derivative mercapturic acid conjugate.

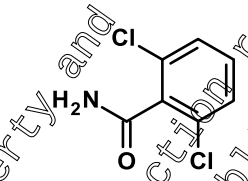
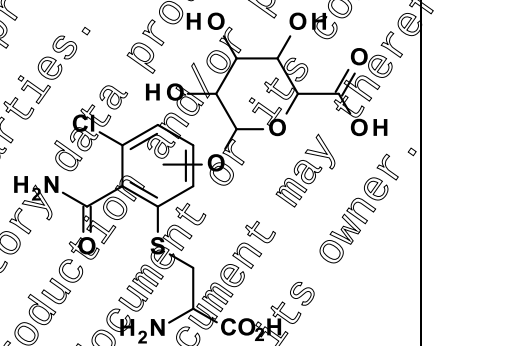
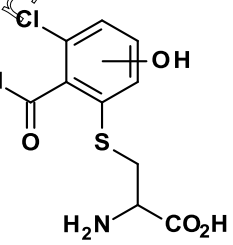
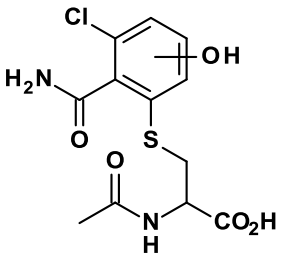
The second proposed metabolic pathway leading to URLD/9 was the aromatic hydroxylation of the test item leading to a hydroxy metabolite (intermediate 1, identified in M-218352-01-1) that was subsequently dehalogenated, followed by the action of GSH enzymes, and the loss of glutamic acid and glycine to obtain URLD/6. The metabolite URLD/6 was subsequently N-acetylated leading to its derivative mercapturic acid conjugate URLD/9. The metabolic pathway provides further details of the two biotransformation pathways leading to the mercapturic acid conjugate.

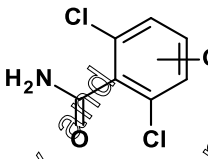
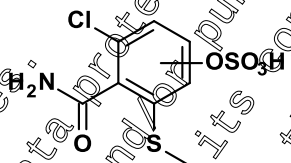
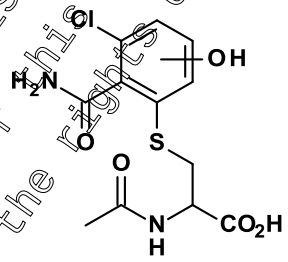
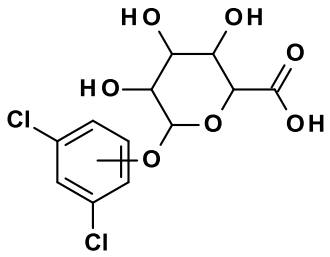
The metabolite URLD/6 accounted for 4.20% in male rats and 12.36% in female rats. URLD/6 was further metabolised by the action of glucuronide enzymes leading to URLD/3 the O-glucuronide conjugate of URLD/6. The metabolite URLD/3 accounted for a total of 3.36% in male urine and 5.19% in female urine. A different biotransformation of URLD/6 was obtained by the action of S-dealkylation enzymes (loss of aryl cysteine) leading to thiomethyl hydroxy-chlorobenzamide (intermediate 3, identified in M-218352-01-1) which was subsequently metabolised by the action of sulfatase enzymes leading to O-sulphate conjugate of thiomethyl-chlorobenzamide (URLD/10b).

The metabolite URLD/10 amounted to a total of 5.47% in male urine and 5.44% in female urine. Spectral data of URLD/10 showed the presence of a shoulder peak that was identified to be the O-sulphate conjugate derived from the hydroxy-dichlorobenzamide metabolite (URLD/10a). The metabolic pathway was completed with the identification of two more products that resulted from the decarboxylation of derived metabolites, one of them was the O-glucuronide conjugate of dichlorobenzamide (URLD/19) and the other one was the O-sulphate conjugate of dichlorobenzamide (URLD/19). The presence of intermediates 1, 2, 3 and 4 was confirmed from samples of the single oral high dose study (M-218352-01-1).

The following table presents a summary of the identified metabolites. The levels of M-01 and it metabolites quantified in urine and faeces are provided in tables 5.1.1-80 and 5.1.1-81, for male and female rats, respectively.

Table 5.1.1- 79: Identified metabolites

Report name (peak ID in the profiles)	Chemical name (IUPAC)	Chemical Structure
Test item M-01 (BAM, AE C653711, BCS-AA65784) peak ID in the profiles: URLD/18 and FRLD/11	2,6-dichlorobenzamide	
URLD/3 Cysteine and O-glucuronide conjugate of chlorobenzamide	Depending on the position of the glucuronide conjugation IUPAC: 6-[4-(2-amino-2-carboxy-ethyl)sulfanyl-3-carbamoyl-2-chloro-phenoxy]-3,4,5-trihydroxy-tetrahydropyran-2-carboxylic acid Or 6-[3-(2-amino-2-carboxy-ethyl)sulfanyl-4-carbamoyl-5-chloro-phenoxy]-3,4,5-trihydroxy-tetrahydropyran-2-carboxylic acid Or 6-[2-(2-amino-2-carboxy-ethyl)sulfanyl-3-carbamoyl-4-chloro-phenoxy]-3,4,5-trihydroxy-tetrahydropyran-2-carboxylic acid	
URLD/6 Cysteine conjugate of hydroxy-chlorobenzamide	Depending on the position of the hydroxylation IUPAC: 2-amino-3-(2-carbamoyl-3-chloro-5-hydroxy-phenyl)sulfanyl-propanoic acid Or 2-amino-3-(2-carbamoyl-3-chloro-6-hydroxy-phenyl)sulfanyl-propanoic acid Or 2-amino-3-(2-carbamoyl-3-chloro-4-hydroxy-phenyl)sulfanyl-propanoic acid	
URLD/9 Mercapturic acid conjugate of hydroxy-chlorobenzamide Isomer of URLD/13 & URLD/16	Depending on the position of the hydroxylation IUPAC: 2-acetamido-3-(2-carbamoyl-3-chloro-5-hydroxy-phenyl)sulfanyl-propanoic acid Or 2-acetamido-3-(2-carbamoyl-3-chloro-6-hydroxy-phenyl)sulfanyl-propanoic acid Or 2-acetamido-3-(2-carbamoyl-3-chloro-4-hydroxy-phenyl)sulfanyl-propanoic acid	

Report name (peak ID in the profiles)	Chemical name (IUPAC)	Chemical Structure
URLD/10 a O-Sulfate conjugate of dichlorobenzamide	Depending on the position of the O-sulfate conjugation IUPAC: (4-carbamoyl-3,5-dichloro-phenyl) hydrogen sulfate Or (3-carbamoyl-2,4-dichloro-phenyl) hydrogen sulfate	
URLD/10 b O-Sulfate conjugate of thiomethyl-chlorobenzamide	Depending on the position of the O-sulfate conjugation IUPAC: (4-carbamoyl-3-chloro-5-methylsulfanyl-phenyl) hydrogen sulfate Or (3-carbamoyl-2-chloro-4-methylsulfanyl-phenyl) hydrogen sulfate Or (3-carbamoyl-4-chloro-2-methylsulfanyl-phenyl) hydrogen sulfate	
URLD/13 Mercapturic acid conjugate of hydroxy-chlorobenzamide Isomer of URLD/9 & URLD/16	Depending on the position of the hydroxylation IUPAC: 2-acetamido-3-(2-carbamoyl-3-chloro-5-hydroxy-phenyl)sulfanyl-propanoic acid Or 2-acetamido-3-(2-carbamoyl-3-chloro-6-hydroxy-phenyl)sulfanyl-propanoic acid Or 2-acetamido-3-(2-carbamoyl-3-chloro-4-hydroxy-phenyl)sulfanyl-propanoic acid	
URLD/15 O-Glucuronide conjugate of dichlorophenyl	Depending on the position of the glucuronide conjugation IUPAC: 6-(2,4-dichlorophenoxy)-3,4,5-trihydroxy-tetrahydropyran-2-carboxylic acid Or 6-(3,5-dichlorophenoxy)-3,4,5-trihydroxy-tetrahydropyran-2-carboxylic acid	

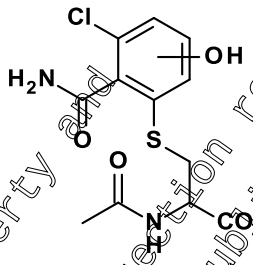
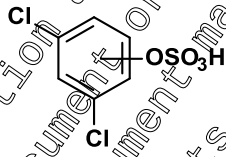
Report name (peak ID in the profiles)	Chemical name (IUPAC)	Chemical Structure
URLD/16 Mercapturic acid conjugate of hydroxy-chlorobenzamide Isomer of URLD/9 & URLD/13	Depending on the position of the hydroxylation IUPAC: 2-acetamido-3-(2-carbamoyl-3-chloro-5-hydroxy-phenyl)sulfanyl-propanoic acid Or 2-acetamido-3-(2-carbamoyl-3-chloro-6-hydroxy-phenyl)sulfanyl-propanoic acid Or 2-acetamido-3-(2-carbamoyl-3-chloro-4-hydroxy-phenyl)sulfanyl-propanoic acid	
URLD/19 O-Sulfate conjugate of dichlorophenyl	Depending on the position of the O-sulfate conjugate IUPAC: (3,5-dichlorophenyl) hydrogen sulfate Or (2,4-dichlorophenyl) hydrogen sulfate	

Table 5.1.1- 80: Balance of [phenyl-UL-^{14}C]-M-01 (BAM) and metabolites excreted after repeated oral administration to male rats (expressed as % dose administered)

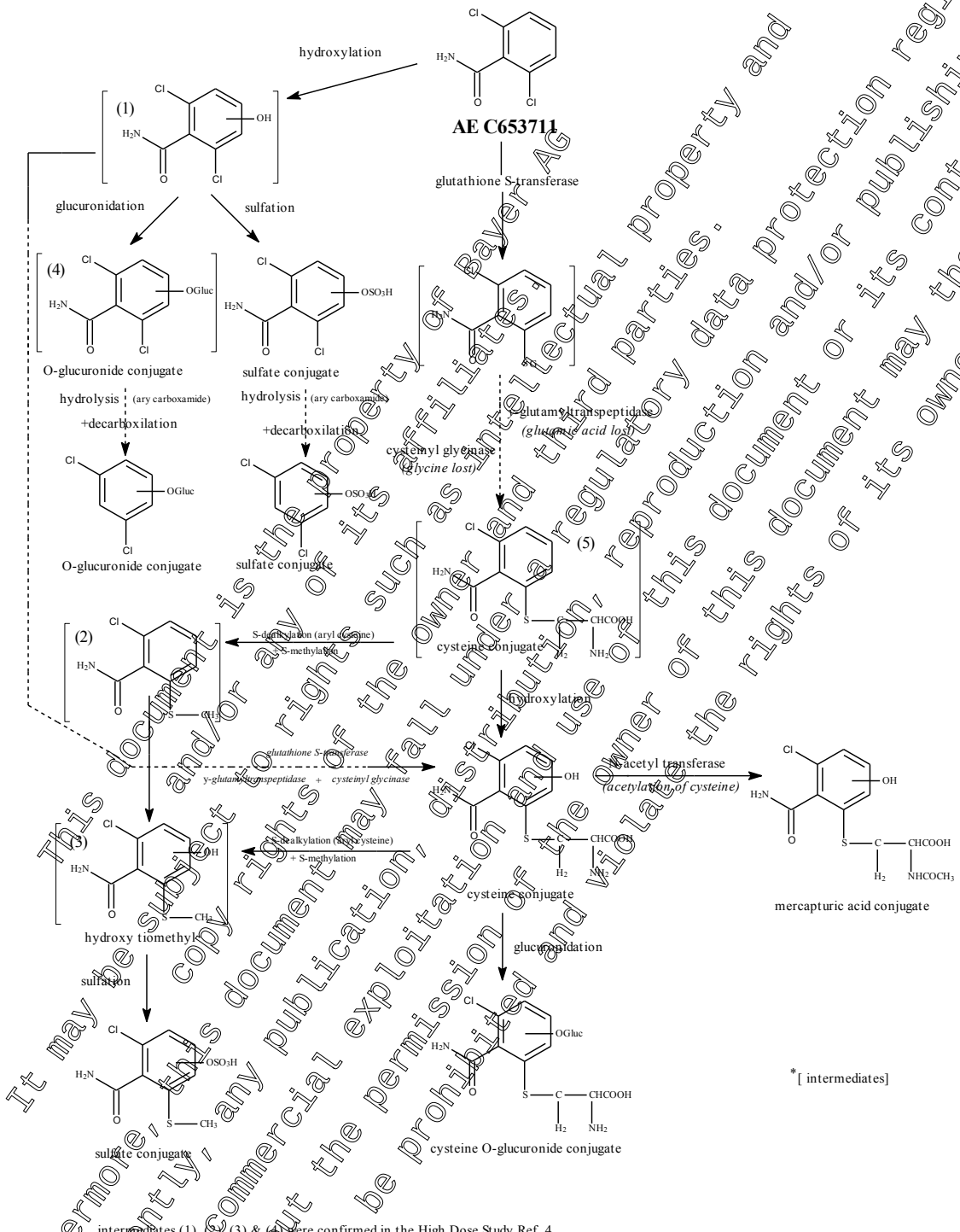
Peak ID	Name	Test 1 (male, 10 mg/kg bw)					
		Day 1			Day 14		
		Urine	Faeces	Total	Urine	Faeces	Total
		% of dose administered					
URLD/3	Cysteine and O-glucuronide conjugate of chlorobenzamide	2.105	-	2.105	3.364	-	3.364
URLD/6	Cysteine conjugate of hydroxy-chlorobenzamide	2.609	-	2.609	4.202	-	4.202
URLD/9	Mercapturic acid conjugate of hydroxy-chlorobenzamide	12.871	-	12.871	15.476	-	15.476
URLD/10	O-Sulfate conjugate of dichlorobenzamide O-Sulfate conjugate of thiomethyl-chlorobenzamide	4.096	-	4.096	5.473	-	5.473
URLD/13	Mercapturic acid conjugate of hydroxy-chlorobenzamide	0.507	-	0.507	0.766	-	0.766
URLD/15	O-Glucuronide conjugate of dichlorophenyl	0.225	-	0.225	0.168	-	0.168
URLD/16	Mercapturic acid conjugate of hydroxy-chlorobenzamide	0.168	-	0.168	0.202	-	0.202
URLD/13, FRLD/11	M-01	2.777	4.593	7.370	9.490	10.404	19.894
URLD/19	O-Sulfate conjugate of dichlorophenyl	0.337	-	0.337	0.705	-	0.705
Total identified		25.695	4.593	30.288	39.846	10.404	50.250
URLD/1	Unknown	0.386	-	0.386	0.491	-	0.491
URLD/2	Unknown	0.370	-	0.370	0.662	-	0.662
URLD/4	Unknown	0.992	-	0.992	1.656	-	1.656
URLD/5	Unknown	0.424	-	0.424	0.391	-	0.391
URLD/7	Unknown	0.430	-	0.430	0.821	-	0.821
URLD/8	Unknown	0.207	-	0.207	0.280	-	0.280
URLD/11	Unknown	0.348	-	0.348	2.204	-	2.204
URLD/12	Unknown	0.281	-	0.281	0.693	-	0.693
URLD/14	Unknown	n.d.	-	n.d.	0.185	-	0.185
URLD/17	Unknown	0.089	-	0.089	0.216	-	0.216
FRLD/1	Unknown	-	0.194	0.194	-	1.621	1.621
FRLD/2	Unknown	-	0.111	0.111	-	0.677	0.677
FRLD/3	Unknown	-	0.080	0.080	-	0.542	0.542
FRLD/4	Unknown	-	0.631	0.631	-	0.692	0.692
FRLD/5	Unknown	-	0.278	0.278	-	1.029	1.029
FRLD/6	Unknown	-	1.966	1.966	-	0.597	0.597
FRLD/7	Unknown	-	0.446	0.446	-	n.d.	n.d.
FRLD/8	Unknown	-	0.401	0.401	-	0.185	0.185
FRLD/9	Unknown	-	0.378	0.378	-	0.187	0.187
FRLD/10	Unknown	-	0.305	0.305	-	0.284	0.284
FRLD/12	Unknown	-	0.258	0.258	-	0.731	0.731
Total characterized		3.527	5.048	8.575	7.599	6.545	14.144

Table 5.1.1- 81: Balance of [phenyl-UL-^{14}C]-M-01 and metabolites excreted after repeated oral administration to female rats (expressed as % dose administered)

Peak ID	Name	Test 1 (female, 10 mg/kg bw)					
		Day 1			Day 14		
		Urine	Faeces	Total	Urine	Faeces	Total
		% of dose administered					
URLD/3	Cysteine and O-glucuronide conjugate of chlorobenzamide	2.092	-	2.092	5.187	-	5.187
URLD/6	Cysteine conjugate of hydroxy-chlorobenzamide	5.699	-	5.699	12.358	-	12.358
URLD/9	Mercapturic acid conjugate of hydroxy-chlorobenzamide	11.159	-	11.159	16.004	-	16.004
URLD/10	O-Sulfate conjugate of dichlorobenzamide O-Sulfate conjugate of thiomethyl-chlorobenzamide	3.983	-	3.983	5.444	-	5.444
URLD/13	Mercapturic acid conjugate of hydroxy-chlorobenzamide	0.580	-	0.580	1.038	-	1.038
URLD/15	O-Glucuronide conjugate of dichlorophenyl	0.216	-	0.216	0.324	-	0.324
URLD/16	Mercapturic acid conjugate of hydroxy-chlorobenzamide	0.332	-	0.332	0.606	-	0.606
URLD/13, FRLD/11	M-01	4.030	4.827	8.857	11.717	7.744	19.461
URLD/19	O-Sulfate conjugate of dichlorophenyl	0.466	-	0.466	0.807	-	0.807
Total identified		28.557	4.827	33.384	53.485	7.744	61.229
URLD/1	unknown	0.355	-	0.355	0.564	-	0.564
URLD/2	unknown	0.329	-	0.329	0.737	-	0.737
URLD/4	unknown	1.608	-	1.608	2.932	-	2.932
URLD/5	unknown	0.583	-	0.583	0.692	-	0.692
URLD/7	unknown	0.532	-	0.532	0.785	-	0.785
URLD/8	unknown	0.258	-	0.258	0.356	-	0.356
URLD/11	unknown	0.431	-	0.431	3.630	-	3.630
URLD/12	unknown	0.235	-	0.235	0.673	-	0.673
URLD/14	unknown	n.d.	-	n.d.	n.d.	-	n.d.
URLD/17	unknown	0.157	-	0.157	0.227	-	0.227
FRLD/1	unknown	-	0.423	0.423	-	0.398	0.398
FRLD/2	unknown	-	0.410	0.410	-	0.304	0.304
FRLD/3	unknown	-	0.324	0.324	-	0.866	0.866
FRLD/4	unknown	-	0.508	0.508	-	0.730	0.730
FRLD/5	unknown	-	1.068	1.068	-	1.921	1.921
FRLD/6	unknown	-	0.476	0.476	-	0.764	0.764
FRLD/7	unknown	-	n.d.	n.d.	-	n.d.	n.d.
FRLD/8	unknown	-	0.581	0.581	-	0.702	0.702
FRLD/9	unknown	-	0.504	0.504	-	0.448	0.448
FRLD/10	unknown	-	0.500	0.500	-	0.470	0.470
FRLD/12	unknown	-	0.721	0.721	-	0.614	0.614
Total characterised		4.488	5.515	10.003	10.596	7.217	17.813

The following metabolic pathway is proposed:

Figure 5.1.1-19: Proposed Metabolic Pathway for [^{14}C]-M-01 following repeated oral dosing at the rate of 10 mg/kg bw/d



III. Conclusions

Overall, multiple dosing of M-01 did not have any significant impact in the absorption, distribution, metabolism, and elimination compared to results after single oral dosing. The routes and rates of excretion were maintained with multiple dosing, with most of the radioactivity eliminated *via* the urinary route within 72 hours post multiple dosing. The distribution pattern in the tissues was similar between the males and females albeit with higher levels being observed for the males. In terms of concentration the males were found to possess tissue levels that were a mean of 6.5 times (±2.5 times) higher than those observed following a single oral low dose of 10 mg/kg ([redacted] 2003; M-218350-01-1), whilst the females displayed levels that were a mean of 3.1 times (±1.9) those observed following a single oral low dose. As these increases were less than half the increase in the amounts of administered product, it would appear that M-01 was not subject to bioretention. Moreover, in terms of % dose, the proportion of radioactivity remaining in the tissues 144 hours after the last of 14 daily administrations of [^{14}C]-M-01 was lower than that observed 144 hours following a single oral low dose. Thus, the mean values in tissues after single oral dose were 2.21% for males and 1.71% for females, whilst the mean values in tissues after repeat dose were 1.07% for males and 0.69% for females. The metabolism of M-01 after multiple dosing was the same compared to single oral dosing, where the biotransformation leading to the mercapturic acid conjugate was the principal metabolic pathway for elimination of the test product. The metabolism of M-01 was completed with the excretion of several O-sulphate and O-glucuronide conjugates that were mainly eliminated in urine. The unchanged test item was seen in urine and faecal samples.

Assessment and conclusion by applicant:
An acceptable study yielding valid conclusions.

Data Point:	KCA 5.1.1/17
Report Author:	[redacted]
Report Year:	2002
Report Title:	Single oral low dose A.D.M.E. study [Pyridyl-2,6-14C]-AE C657188 (PCA)
Report No:	SA 01093
Document No:	M-217250-01-1
Guideline(s) followed in study:	EU 87/302/EEC Part B; JMAFF: 59 NohSan 4200; US-EPA OPPTS 870.7485
Deviations from current test guideline:	None
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary

The absorption, distribution, metabolism and excretion of [pyridyl-2,6-^{14}C]-AE C657188 (PCA, or M-02) was investigated in rats following a single oral gavage dose at a nominal 10 mg/kg bw.

A group of 4 male and 4 female Sprague-Dawley rats received a single achieved dose of approximately 11 mg/kg bw by oral gavage. Urine, faeces and expired air were sampled at up to 120 h after dosing, at which time the animals were sacrificed and tissues and blood were sampled.

Recovery over the five day period after dosing was found to be 94.50% for males, 92.74%, for females. The rate of elimination was rapid and similar for both male and female rats with at least 90% of the total administered radioactivity eliminated within the first 48 hours post dose. No pulmonary excretion was detected. With regard to tissues, radioactivity was detected only in the residual carcass and in the skin and fur, together representing only 0.23% and 0.30% of administered dose for males and females respectively.

Estimated minimum absorption levels (sum of residues in urine, cagewash and tissues) were 86.9% for males and 87.1% for females.

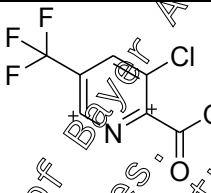
Chromatographic analysis showed that the parent was the major component excreted in urine and faeces, accounting for 86% and 79% of the administered dose in males and females respectively. All other fractions in the urine represented <0.16%, except for one that represented 1.38% of the dose, while in faeces the two remaining metabolites did not represent more than 0.10% of the dose.

It was concluded that a high oral bioavailability and low potential for bioaccumulation had been demonstrated. The parent M-02 was subject to only a minor amount of metabolism and was the major component excreted in urine and faeces, with no detected excretion in expired air.

I. Materials and methods

A. Materials

1. Test material

IUPAC Name	3-chloro-5-(trifluoromethyl)pyridine-2-carboxylic acid
Code name	AE C657188 or M-02
Common name	Pyridine carboxylic acid (PCA)
Empirical formula	$C_7H_3ClF_3NO_2$
Molar mass	225.58 g/mol
Chemical structure	 + *denotes positions of label*
Radiolabelled test material	[Pyridyl-2,6-^{14}C]-AE C657188
Lot number	DCR25/1
Radiochemical purity	99.0% (TLC and autoradiography)
Specific radioactivity	35.96 mCi/mmole or 1331 MBq/mmole 158.6 µCi/g or 5.87 MBq/mg
Specific radioactivity of dose suspensions	Checked by HPLC (UV detection)
Stability of test compound	Stable at -20°C
Non-labelled test material	AE C657188
Batch numbers	R001739. Also RAW244055/1 (used to complete metabolic ID of residues in excreta)
Purity	99.5% and 97.2% respectively
Storage	-20°C

2. Vehicle: Aqueous methyl cellulose (0.75% w/w)

3. Test animals:

Species: Rat
Strain: Sprague Dawley
Age: Not stated
Weight at dosing: 172.5 – 244.1g
Source: [redacted]
Identification: Ear tattoo
Diet: Certified rodent diet M20 (Pietremont, Provins, France)
Water: Provided *ad libitum* (filtered, softened water from municipal supply)
Housing: Housed in metal wire-mesh bottomed cages in metal trays containing absorbent paper during acclimatisation and in specialised metabolism cages thereafter

Environmental conditions

Temperature:	22 ± 2˚C
Humidity:	55 ± 15%
Photoperiod:	12 hours
Air changes	Average 15 changes/hour

4. Preparation of dosing solutions

The doses were prepared by mixing weighed amounts of radiolabelled and non-radiolabelled material in acetonitrile to achieve a homogenous solution at the desired specific radioactivity. The solution was then gently evaporated under a stream of nitrogen, appropriate weights of aqueous methyl cellulose (0.75% w/w) added and the resulting mixture ground with mortar and pestle to produce a suspension. The dose suspension was of sufficient quantity and concentration to provide a dose of 1 g per 200 g body weight. The dose suspensions were prepared on the day before dosing and assayed by HPLC for active ingredient content and for radioactivity content by LSC before, during and following the dosing procedure.

B. Study design and methods

1. In life dates: 28 March 2001 to 11 April 2001

2. Dose regimen and design of test

Radiolabelled test substance	Nominal dose (mg/kg bw, single oral gavage dose)	Rats/sex	Actual mean doses (mg/kg bw)	Specific activity (µCi/mg)	Collection of urine (h)	Collection of faeces and cage-wash (h)	Collection of expired CO_2 (h)
[2,6-pyridyl-^{14}C]-AE C657188	10	4/sex	Males: 10.92 Females: 11.02	13.302	0-6, 6-24, 24-48, 48-72, 72-96, 96-120	0-24, 24-48, 48- 72, 72-96, 96-120	0-6, 6-24, 24-48

3. Dosing

Approximately 18 h prior to dosing, the diet was removed from the cages (access to water was retained), then restored within one hour after dosing. The rats were weighed immediately prior to dosing, which was a single oral dose by gavage.

The rats each received a single dose by oral gavage of labelled fluopicolide at a target low dose of 10 mg/kg bw, with a nominal radioactive dose of 125 µCi/kg. Dose rate was 1 g dose suspension per 200 g body weight.

4. Collection of samples

Excreta samples were collected at the intervals shown, until sacrifice by exsanguination at 120 h (when >90% of the administered radioactivity had been collected). No radioactivity was detected in expired air up to 48 h after dosing and collection was consequently stopped.

The liver, kidneys, heart, lungs, brain, spleen, pancreas, muscle, abdominal fat, ovaries, testes, stomach plus contents, intestine tract plus contents, bone and marrow, adrenals, uterus, eyes, Harderian glands, thyroids, and the skin and fur were removed from each animal. The residual carcass was also retained for analysis. Plasma was prepared from cardiac blood samples by centrifugation.

Wherever possible, samples were processed as they were collected. Otherwise, they were stored at -20°C protected from light until required for analysis.

5. Measurement of radioactivity

The amounts of radioactivity in the various samples were determined by liquid scintillation counting with automatic standard quench correction. The limit of detection was taken to be twice the background values for blank samples in appropriate scintillation cocktails.

Liquid samples were counted in Ultima-gold. Minced, whole or homogenized tissue, blood and faecal samples were combusted, and the trapped CO_2 was mixed in Permafluor for radioassay. Skin, fur, uterus and carcass were solubilize in KOH, then aliquots were added to Hionic Fluor for radioassay.

6. Metabolic investigations

As the urine and faecal samples contained almost the entire recovered radioactivity, the metabolism study was performed with these two categories of samples. Based on the amounts of excreted radioactivity in these samples, the time intervals of 0-6h, 6-24h and 24-48h for male and female urine samples and the interval 0-24h for male and female faecal samples. As there was little individual animal variation, urine samples were pooled according to sex and time period. Pooled samples for the male and female groups: 0-6 hours, 6-24 hours and 24-48 hours were prepared according to the percentage of the recovered radioactivity detected in urine for method development and metabolite identification (*ca* 20% depending on the sample). The pools for 24-48 hours for males and females were concentrated by evaporation under nitrogen.

The pooled homogenate faecal samples were centrifuged and the aqueous supernatants were retained. The remaining pellets were extracted four times with a mixture of acetonitrile/water (80/20 v/v). The organic extracts were pooled and centrifuged and the supernatants were decanted. At this stage 94.45% and 96.27% of the total radioactivity was extracted (aqueous extract plus organic extract). Both extracts (aqueous and organic) were concentrated prior to analysis.

The metabolite profile was investigated by a radio-HPLC technique using three gradient elution systems. Metabolite identification was performed by comparison of retention times with known standards and by LC/MS and LC/MS/MS.

7. Data processing

Calculation of dose recovery and concentrations were carried out using Debra 5.2a and Excel.

II. Results and Discussion

A. Recovery: absorption, distribution and excretion

Recovery over a five day period post-administration was found to be 94.50% for males, 92.74% for females. The rate of elimination was rapid and similar for both male and female rats (sum of radioactivity recovered in the urine, faeces and cage washes) with at least 90% of the total administered radioactivity eliminated within the first 48 hours post dose. No pulmonary excretion was detected.

With regard to tissues, radioactivity was detected only in the residual carcass and in the skin & fur.

Estimated minimum absorption levels (sum of residues in urine, cagewash and tissues) were 86.9% for males and 87.1% for females.

Table 5.1.1- 82: Recovery of radioactivity following 10 mg/kg bw [pyridyl-2,6-^{14}C]-M-02 in rats (% of administered radioactivity/dose)

Sample	Sampling period (h)	Males		Females	
		Mean	SD	Mean	SD
Urine	0-6	55.55	10.95	56.28	5.46
	0-24	77.80	5.84	72.97	9.07
	0-48	79.98	4.72	75.70	9.18
	0-72	80.32	4.54	76.09	9.23
	0-96	80.46	4.46	76.33	9.16
	0-120	80.56	4.39	76.41	9.14
Faeces	0-24	7.24	0.49	5.31	1.86
	0-48	7.35	0.51	5.58	1.85
	0-72	7.45	0.48	5.63	1.85
	0-96	7.51	0.50	5.64	1.84
	0-120	7.59	0.55	5.68	1.87
Cage Wash	0-120	6.12	3.34	10.36	7.08
CO_2 trap	0-48	n.d.	n.a.	n.d.	n.a.
Tissues	0-120	0.23	0.06	0.30	0.04
Total	0-120	94.50	1.13	92.74	2.04
Skin+fur[a]	0-120	0.062	0.022	0.092	0.031
Carcass[a]	0-120	0.021	0.004	0.025	0.006
Absorption[b]		86.91		87.07	

SD = Standard Deviation. n.d. = not detected n.a. = not applicable
a Expressed as µg equivalents/g
b Sum of urine, cagewash and tissues

B. Metabolite quantification

A total of 9 components were detected on the chromatograms for the urine. They were numbered U/1 to U/9 according to their order of appearance, U/9 being the least polar with the longest retention time. Three components were observed in the faeces, similarly designated F/1 to F/3.

Table 5.1.1- 83: Proportion of administered dose in urine following 10 mg/kg bw [pyridyl-2,6-^{14}C]-M-02 in male rats (% of administered dose)

Metabolite	0-6 h	6-24 h	24-48 h	Total
U/1	0.02	n.d.	n.d.	0.02
U/2	0.04	n.d.	n.d.	0.04
U/3	n.d.	n.d.	n.d.	n.d.
U/4	n.d.	0.02	n.d.	0.02
U/5	54.53	21.84	2.18	78.85
U/6	0.47	0.26	n.d.	0.73
U/7	0.09	0.07	n.d.	0.16
U/8	0.01	0.02	n.d.	0.03
U/9	0.11	0.03	n.d.	0.14
Total	55.57	22.24	2.18	79.99

Table 5.1.1- 84: Proportion of administered dose in urine following 10 mg/kg bw [pyridyl-2,6-^{14}C]-M-02 in female rats (% of administered dose)

Metabolite	0-6 h	6-24 h	24-48 h	Total
U/1	0.01	n.d.	n.d.	0.01
U/2	n.d.	n.d.	n.d.	n.d.
U/3	0.03	n.d.	n.d.	0.03
U/4	0.02	0.04	n.d.	0.06
U/5	54.86	16.36	2.72	73.94
U/6	1.17	0.19	0.02	1.38
U/7	0.07	0.07	n.d.	0.14
U/8	n.d.	n.d.	n.d.	n.d.
U/9	0.11	0.03	n.d.	0.14
Total	56.27	16.69	2.74	75.70

Table 5.1.1- 85: Proportion of administered dose in faeces following 10 mg/kg bw [pyridyl-2,6-^{14}C]-M-02 in male and female rats (% of administered dose)

Metabolite	Males 0-24 h	Females 0-24 h
F/1	0.17	0.10
F/2	7.01	5.19
F/3	0.06	0.03
Total	7.24	5.32

Overall, the results showed that in the excreta (urine and faeces) there was one major component, accounting for 86% and 79% of the administered dose in males and females respectively. All other fractions in the urine represented <0.16%, except for one that represented 1.38% of the dose, while in faeces the two remaining metabolites did not represent more than 0.10% of the dose.

III. Conclusions

The high levels of radioactivity in urine plus cage washes demonstrated a high oral bioavailability and low potential for bioaccumulation. The parent M-02 was subject to only a minor amount of metabolism and was the major component excreted in urine and faeces, with no detected excretion in expired air.

Assessment and conclusion by applicant

An acceptable study yielding valid conclusions

Data Point:	KCA 5.1.1.18
Report Author:	[redacted]
Report Year:	2019
Report Title:	Amendment no. 01: Interspecies comparison of in vitro metabolism of [phenyl-UL-14C] fluopicolide using mouse, rat, dog and human liver microsomes
Report No:	20157742
Document No:	M-653630-02-1
Guideline(s) followed in study:	Regulation EU 283/2013.
Deviations from current test guideline:	None
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

The objective of this study was to determine the metabolic stability and metabolite profiles of [phenyl-UL-^{14}C] fluopicolide in liver microsomes from mixed-gender CD1 mice, Wistar rats, Beagle dogs and humans.

The [^{14}C]-test item was incubated at 1 and 10 µM. Incubation with 1 µM was used for metabolic stability calculations and incubation with 10 µM was used for metabolite profiling purposes. Incubations with [^{14}C]-test item were performed for 1, 60 and 120 (±1) minutes in duplicate for all species.

An LC-PDA-RAD method was used for the analysis of incubated samples. The method was found to be adequate and the chemical stability of the test item under the conditions used was determined.

The metabolic activity of the liver microsomal batch was confirmed by measuring one CYP-dependent enzymatic activity (phenacetin metabolism).

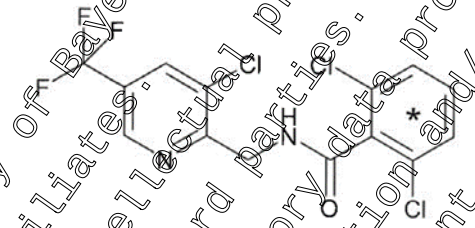
The [^{14}C]-test item showed significant metabolism in all species. The extent of conversion of [^{14}C]-test item was 98% in dog, 82% in mouse, 68% in human and 54% in rat liver microsomes after 120 minutes of incubation. The calculated *in vitro* $t_{1/2}$ and intrinsic clearance $CL'_{int, in\ vitro}$ values were 48 & 26, 91 & 11, 26 & 53, 73 & 7 in mouse, rat, dog and human, respectively.

A total of 8 metabolites were found in the different incubations of which M1, M2, M3, M5 and M6 represented more than 5% of the total radioactivity. Metabolite M2 was unique to mice and rats. No human specific metabolites were detected.

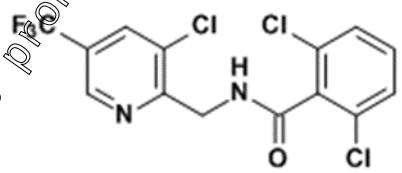
I. Materials and Methods

A. Materials

1. Test material

Radiolabelled test material	[phenyl-UL-^{14}C] Fluopicolide
Chemical structure	 *denotes position of label*
Lot number	KML 10634
Radiochemical purity	>98%
Chemical purity	>99%
Specific activity	3.53 MBq/mg

2. Reference item

IUPAC Name	2,6-dichloro-N-[[3-chloro-5-(trifluoromethyl)-2-pyridinyl]methyl]-benzamide
CAS Name	benzamide, 2,6-dichloro-N-[[3-chloro-5-(trifluoromethyl)-2-pyridinyl]methyl]-
Code name	AE C638206
Common name	Fluopicolide
Empirical formula	$C_{14}H_8Cl_3F_3N_2O$
CAS Number	239110-15-7
Molar mass	383.6 g/mol
Chemical structure	
Lot number	AE C638206 00 1B99 003

3. Test system

Test system	Liver microsomes form mixed gender CD1 mouse, Wistar rat, Beagle dog and human
Rationale	Liver microsomes have Cytochrome P450 (Phase I) activities.
Source	[redacted]
Batch/Lot	CD-1 mouse: YSW (male) and MDO (female) Wistar rat: LTH (male) and VRM (female) Beagle dogs: HSN (male) and GOV (female) Human (SNB (mixed gender)
Storage	The liver microsomes were stored in the ultra-low freezer (≤-75°C).

B. Study design and methods

1. Experimental phase

September 03 to September 22, 2018

2. Experimental procedures

Preparation of stock solutions

Stock solutions of the supplied [phenyl-UL-^{14}C]-fluopicolide were prepared in acetonitrile (ACN), the activity of the stock solutions was determined by liquid scintillation counting and a final activity concentration of 6.69 MBq/mL was achieved. Incubation mixtures were prepared at a final concentration 1 and 10 mM.

Pre-experiments

Prior to the main study, an LC-PDA-RAD method was optimised, and pilot incubation experiments were performed with rat liver microsomes. Additionally, the linearity and chemical stability of the test item under incubation conditions was confirmed. Adequate recovery efficiencies (>85%) were achieved.

Main study

For each species duplicate incubations were performed with [phenyl-UL-^{14}C]-fluopicolide(1 and 10 µM final concentration) in incubation buffer containing liver microsomes at different time-points. Phenacetin (30 µM final concentration) in incubation buffer containing liver microsomes at two different time-points acted as the phase I metabolic activity control (control 1). All incubations were subject to LC-PDA-RAD analysis. Samples containing 1 µM were used for metabolic stability calculations and samples containing 10 µM of test item were used for metabolic profiling purposes.

Radioactivity in all samples was determined by liquid scintillation counting (LSC).

Microsomal incubations

Duplicate incubation mixtures with [14C]-test item (1 and 10 µM final concentration) were prepared in glass vials closed with a screw cap by mixing as follows:

(1) 990 µL of 0.1 M potassium phosphate buffer pH 7.4

(2) 400 µL 2.5 mg/mL liver microsomes in 0.1 M potassium phosphate buffer pH 7.4

(3) 400 µL 5 mM NADPH in 0.1 M potassium phosphate buffer pH 7.4

(4) 200 µL 100 mM G6P with 100 U/mL G6P-DH in 0.1 M potassium phosphate buffer pH 7.4

After pre-incubation for 5±1 minutes at 37±1°C in a water bath, 10 µL of the [14C] test item Spiking 200 or Spiking 2000 solution was added resulting in final [14C] test item concentrations of 1 µM and 10 µM, respectively, in a 0.5% (v/v) aqueous solution of ACN.

Each incubation mixture was divided into three vials of 400 µL each and, from the remaining mixtures, an aliquot of 20 µL per sample was transferred to a scintillation vial and counted by LSC, after addition of 10 mL Ultima Gold cocktail.

The vials were incubated in a shaking water bath at 37±1°C. After 1±1, 60±1 and 120±1 minutes of incubation, vials were cooled down on ice, and 200 µL ACN was added. After vortex-mixing, the samples were centrifuged for 5 minutes at 16,000 g. Of each supernatant, 20 µL was transferred to scintillation vials containing 10 mL Ultima Gold cocktail and counted by LSC to determine the radioactivity in the supernatant. The rest of the supernatant was transferred to a vial and subjected to LC-PDA-RAD analysis. Samples were stored in the freezer (≤ -15°C) after analysis. The remaining pellets were stored in the freezer (≤ 15°C).

Metabolic activity controls

The metabolic competence of each of the microsomal preparations was assessed in duplicate, in parallel with the [14C]-test item incubations, by measuring the rate of phenacetin (30 µM) metabolism.

Metabolic competence in liver microsomes was determined in duplicate by incubating:

(1) 295 µL 0.1 M potassium phosphate buffer pH 7.4

(2) 50 µL 2.5 mg/mL liver microsomes in 0.1 M potassium phosphate buffer pH 7.4

(3) 100 µL 5 mM NADPH in 0.1 M potassium phosphate buffer pH 7.4

(4) 50 µL 100 mM G6P with 100 U/mL G6P-DH in 0.1 M potassium phosphate buffer pH 7.4

After pre-incubation for 5 minutes at 37±1°C, 5 µL of 3 mM phenacetin was added. After 1±1, 60 ±1 and 120 ±1 minutes of incubation, 100 µL was withdrawn and diluted with 100 µL of incubation buffer. The metabolic activity samples were stored in the freezer (≤ 15°C) for a maximum of 1 month. Prior to analysis the samples were thawed, centrifuged for 5 minutes at 16,000 g and subjected to UPLC PDA MS analysis.

Phenacetin (enzyme activity: phenacetin O-deethylase) was used as positive control for CYP1A2 activity (=Phase I metabolism).

3. Analysis

Metabolic activity

The metabolic activity of the liver microsomes was evaluated by determining the formation of detectable amounts of acetaminophen from phenacetin and by determining the disappearance of phenacetin expressed as percentage of the start concentration, based on peak area and conversion rate.

Metabolic Stability of the [^{14}C]-Test Item

The percentage [phenyl-UL-^{14}C] Fluopicolide remaining was defined as the ratio of the [^{14}C]-test item peak area at a specific time point and the peak area in the corresponding incubation samples at t=1 minute multiplied by 100%.

The metabolic stability was evaluated from the 1 µM incubations by plotting the natural logarithm of the percentage [^{14}C]-test item remaining versus time and performing linear regression. From the slope the elimination constant k_e was calculated (k_e=-slope). From k_e the in vitro half-life $t_{1/2}$ was calculated ($t_{1/2}=\ln(2)/k_e$). Metabolic stability was compared across species.

Metabolite Profile of the [^{14}C]-Test Item

Using RAD chromatograms, the formation of metabolites (expressed as % of total radioactivity eluted in each sample) was evaluated.

II. Results and Discussion

A. Results

The mean radiochemical purity of [phenyl-UL-^{14}C]-fluopicolide was determined to be 99.5% and a peak retention time of 19.2-19.3 minutes was determined, which was comparable to the unlabelled fluopicolide, thus confirming the identity of the radiolabelled material.

The recovery of [phenyl-UL-^{14}C]-fluopicolide from the incubation media was determined to be 102% using LC-RAD; the analytical method was considered suitable for determining changes in concentration in the incubated sample, owing to the linear relationship between response and [phenyl-UL-^{14}C]-fluopicolide concentration on the range of 0.1-10 µM when using RAD.

It was determined that [phenyl-UL-^{14}C]-fluopicolide was stable under the microsomal conditions used as the peak area did not decrease after incubations for 120 minutes at 37˚C. Furthermore, the recovery of radioactivity of the chemical stability samples was 101-109%.

The microsomal batches of all species metabolised phenacetin, thus confirming that all batches were metabolically active. The peak area of phenacetin in Wistar rat control incubations increased at t=120 minutes; however, acetaminophen was formed thus demonstrating metabolic activity.

The results from the 1 µM and 10 µM microsomal incubations are presented below. The 1 µM incubations were used for evaluation of metabolic stability and intrinsic clearance calculations, whilst the 10 µM incubations are presented for comparison purposes, indicating stability at higher concentrations.

The average extent of conversion of [phenyl-UL-^{14}C]-fluopicolide after 120 minutes' incubation was 98%, 82%, 68% and 54% in dog, mouse, human and rat microsomes, respectively. The calculated *in vitro* $t_{1/2}$ values were 26, 48, 73 and 91 minutes in the same respective incubations.

The recovery of radioactivity in the microsomal samples after pre-treatment was ≥85% in all species, except for one replicate of the rat t=120 minute sample (83%); this sample was therefore rejected form the calculations.

Table 5.1.1- 86: Mean recovery of radioactivity in mouse, rat, dog, and human microsomal incubation samples with [phenyl-UL-^{14}C]-fluopicolide after sample pre-treatment

Species	Time-point (min)	[phenyl-UL-^{14}C]-fluopicolide	
		1 µM	10 µM
Mouse	1	102	98
	60	91	96
	120	90.5	93.5
Rat	1	94	97
	60	94.5	100
	120	87.5	95
Dog	1	92.5	96.5
	60	90.5	94.5
	120	88.5	89.5
Human	1	96.5	98
	60	94.5	93.5
	120	91	91

All microsomal incubation samples were analysed by LC-PDA-RAD. [phenyl-UL-^{14}C]-fluopicolide was metabolised into 5-8 metabolites in the liver microsomal incubations from the different species. After 120 minutes of incubation, [phenyl-UL-^{14}C]-fluopicolide was on average 74.4, 44.1, 40.4 and 7.2% as total radioactivity in the chromatogram in the rat, mouse, human and dog incubations samples, respectively.

A total of 8 metabolites were found in the different incubations, 5 of which represented more than 5% of the total radioactivity (M1, M2, M3, M5 and M6). Metabolite M2 was only detected in the rat and mouse incubations, whilst the remaining metabolites were detected in all species. There were no human specific metabolites.

The observed metabolites in the different species are summarised in the table below.

Table 5.1.1- 87: Summary of metabolites of [phenyl-UL-^{14}C]-fluopicolide with liver microsomal incubations of different species

Retention time	RRT*	Metabolite	Replicate	% total radioactivity of chromatogram (t=120 mins)			
				CD1 mouse	Wistar rat	Beagle dog	Human
19.1-19.3	0.53	M1	1	14.98	6.74	4.45	16.69
			2	16.03	6.93	4.47	18.34
24.3-24.4	0.67	M2	1	7.75	7.26	-	-
			2	8.57	6.62	-	-
29.2-29.5	0.81	M3	1	6.29	0.75	22.73	22.52
			2	7.71	0.83	25.85	24.40
32.2-32.4	0.89	M4	1	-	-	1.34	-
			2	-	-	0.77	-
33.5-33.6	0.93	M5	1	9.37	2.00	15.45	8.25
			2	9.53	2.08	14.30	9.38
34.7-34.9	0.95-0.96	M6	1	15.47	7.95	44.62	8.80
			2	15.10	8.88	46.97	9.65
35.5	0.97	M7	1	-	-	-	-
			2	-	-	-	-
36.2-36.5	1.00	T1	1	45.62	74.70	7.48	43.28
			2	42.52	74.04	6.85	37.60
37.2-37.3	1.02-1.03	M8	1	0.47	0.59	0.94	0.46
			2	0.54	0.61	0.78	0.62

*relative retention time compared with parent compound

III. Conclusions

[Phenyl-UL-^{14}C]-fluopicolide was metabolised in CD1 mice, Wistar rat, Beagle dog and human liver microsomal incubations, the extent of conversion was 82%, 54%, 98% and 68% respectively after 120 minutes' incubation and the calculated in vitro $t_{½}$ values were 48, 91, 26 and 73 minutes respectively.

A total of 8 metabolites were found, of which 5 represented more than 5% of the total radioactivity in at least one species (M1, M2, M3, M5 and M6).

No human specific metabolites were detected.

Assessment and conclusion by applicant:
Acceptable study yielding valid conclusions.

Data Point:	KCA 5.1.1/19
Report Author:	[redacted]
Report Year:	2020
Report Title:	In vitro metabolism of [phenyl-UL-14C] fluopicolide using rabbit liver microsomes
Report No:	20224451
Document No:	M-685653-01-1
Guideline(s) followed in study:	EU Commission Regulation (EC) No 283/2013 (March 2013)
Deviations from current test guideline:	None
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

The objective of this study was to determine the metabolic stability and metabolite profiles of [phenyl-UL-^{14}C] Fluopicolide in liver microsomes from mixed-gender New Zealand White rabbits. The study was intended as a follow up to the previous study ([redacted] 2018; M-653630-02-1), in which the metabolic stability and metabolic profiles of [phenyl-UL-^{14}C] Fluopicolide in liver microsomes from mixed-gender CD1 mice, Wistar rats, Beagle dogs and humans were determined.

The [^{14}C]-test item was incubated at 1 and 10 µM. Incubation with 1 µM was used for metabolic stability calculations and incubation with 10 µM was used for metabolite profiling purposes. Incubations with [^{14}C]-test item were performed for 1, 60 and 120 (n=1) minutes in duplicate.

An LC-PDA-RAD method was used for the analysis of incubated samples. The method was found to be adequate and the chemical stability of the test item under the conditions used was determined in the previous study ([redacted] 2019; M-653630-02-1).

The metabolic activity of the liver microsomal batch was confirmed by measuring one CYP-dependent enzymatic activity (phenacetin metabolism). The remaining percentage of phenacetin was 32% in mixed-gender New Zealand White rabbit liver microsomes after 120 minutes of incubation confirming that the liver microsomal batches used were metabolically active.

The recovery of radioactivity in the microsomal incubations samples after sample pre-treatment was ≥ 94% for the 1 µM samples and ≥ 90% for the 10 µM samples as compared to the spiking solutions used to spike the incubation samples, which is >85% and considered acceptable.

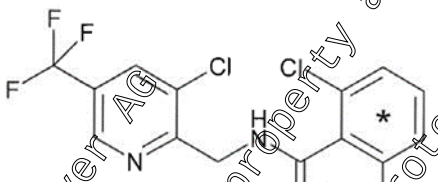
The [^{14}C]-test item showed significant metabolism in mixed-gender New Zealand White Rabbit liver microsomes in which the extent of conversion of [^{14}C]-test item was 90% after 120 minutes of incubation. The calculated *in vitro* $t_{1/2}$ and intrinsic clearance $CL'_{int, in\ vitro}$ values were 36 minutes & 113 (mL/min/kg), respectively.

A total of 10 metabolites were found in the different incubations of which M2, M7, M9 and M10 represented more than 5% of the total radioactivity in the chromatograms.

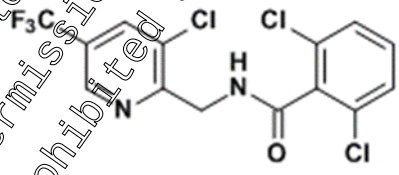
I. Materials and Methods

A. Materials

1. Test material

Radiolabelled test material	[phenyl-UL-^{14}C] Fluopicolide
Chemical structure	 * *denotes position of label*
Lot number	KML 10622
Radiochemical purity	> 99%
Chemical purity	>99%
Specific activity	3.53 MBq/mg

2. Reference item

IUPAC Name	2,6-dichloro-N-[[3-chloro-5-(trifluoromethyl)-2-pyridinyl]methyl]-benzamide
CAS Name	benzamide, 2,6-dichloro-N-[[3-chloro-5-(trifluoromethyl)-2-pyridinyl]methyl]
Code name	AE C638206
Common name	Fluopicolide
Empirical formula	$C_{14}H_8Cl_3F_3N_2O$
CAS Number	239110-15-7
Molar mass	383.6 g/mol
Chemical structure	
Lot number	2016-012208

3. Test system

Test system	Liver microsomes form mixed gender New Zealand White rabbits
Rationale	Liver microsomes have Cytochrome P450 (Phase I) activities.
Source	[redacted]
Batch/Lot	HTO (male) and LOB (female) The single lots were combined in a 1:1 ratio
Storage	The liver microsomes were stored in the ultra-low freezer (≤-75°C).

B. Study design and methods

1. Experimental phase

January 29 to February 03, 2020

2. Experimental procedures

The study comprised the assessment of the radiochemical purity of the [^{14}C]-test item and the investigation of the [^{14}C]-test item metabolism in liver microsomes.

The [^{14}C]-test item was incubated with liver microsomes prepared from the selected species (NZW rabbit). Incubation samples were subjected to LC-PDA-RAD analysis. The metabolite profiles from the LC-PDA-RAD analyses provided information on the relative rates of [^{14}C]-test item metabolism in the rabbit.

Incubations were performed at 1 µM and 10 µM final concentrations. The time course of metabolism was investigated at 1 µM [^{14}C]-test item to estimate clearance, whilst the samples prepared at 10 µM [^{14}C]-test item were used for comparison purposes. Additionally, the samples prepared at 10 µM [^{14}C]-test item provided metabolite profiles by LC-PDA-RAD analysis.

The sample pre-treatment and LC-PDA-RAD method used in the previous metabolic stability study ([redacted] 2019, M-653630-02-1) was used in this study, in which adequate recovery efficiencies, linearity of the method and the chemical stability of the test item under the microsomal incubation conditions was demonstrated. The radiochemical purity of the test item was determined using an LC-UV radioactivity system.

Microsomal incubations

Duplicate incubation mixtures with [14C]-test item (1 and 10 µM final concentration) were prepared in glass vials closed with a screw cap by mixing as follows:

(1) 990 µL of 0.1 M potassium phosphate buffer pH 7.4

(2) 400 µL 2.5 mg/mL liver microsomes in 0.1 M potassium phosphate buffer pH 7.4

(3) 400 µL 5 mM NADPH in 0.1 M potassium phosphate buffer pH 7.4

(4) 200 µL 100 mM G6P with 100 U/mL G6P-DH in 0.1 M potassium phosphate buffer pH 7.4

After pre-incubation for 5±1 minutes at 37±1°C in a water bath, 10 µL of the [14C] test item Spiking 200 or Spiking 2000 solution was added resulting in final [14C] test item concentrations of 1 µM and 10 µM, respectively, in a 0.5% (v/v) aqueous solution of ACN.

Each incubation mixture was divided into three vials of 400 µL each and, from the remaining mixtures, an aliquot of 20 µL per sample was transferred to a scintillation vial and counted by LSC, after addition of 10 mL Ultima Gold cocktail.

The vials were incubated in a shaking water bath at 37±1°C. After 1±1, 60±1 and 120±1 minutes of incubation, vials were cooled down on ice and 200 µL ACN was added. After vortex-mixing, the samples were centrifuged for 5 minutes at 16,000 g. Of each supernatant, 20 µL was transferred to scintillation vials containing 10 mL Ultima Gold cocktail and counted by LSC to determine the radioactivity in the supernatant. The rest of the supernatant was transferred to a vial and subjected to LC-PDA-RAD analysis. Samples were stored in the freezer (≤-15°C) after analysis. The remaining pellets were stored in the freezer (≤ 15°C).

Metabolic activity controls

A 25 mM phenacetin stock solution in ACN was diluted to 3 mM in ACN:MQ 1:3 (v/v) by mixing 60 µL of a 25 mM stock solution with 180 µL MQ and 250 µL ACN:MQ 1:3 (v/v). Both the stock and spiking solutions were stored in the freezer (≤-15°C) for not more than 1 month.

For incubations, the 3 mM spiking solution was diluted 100-fold resulting in final phenacetin concentrations of 30 µM in a 0.25% (v/v) aqueous solution of ACN.

3. Analysis

The amount of radioactivity in the microsomal incubation mixtures at the start of the incubation and in the supernatant at the different time-points was determined by LSC.

Metabolic activity

The metabolic activity of the liver microsomes was evaluated by determining the formation of detectable amounts of acetaminophen from phenacetin and by determining the disappearance of phenacetin expressed as percentage of the start concentration, based on peak area and conversion rate.

Metabolic Stability of the [^{14}C]-Test Item

The percentage [phenyl-UL-^{14}C] Fluopicolide remaining was defined as the ratio of the [^{14}C]-test item peak area at a specific time point and the peak area in the corresponding incubation samples at 1±1 minute multiplied by 100%.

The metabolic stability was evaluated from the 1 µM incubations by plotting the natural logarithm of the percentage [^{14}C]-test item remaining versus time and performing linear regression. From the slope the elimination constant k_e was calculated (k_e=-slope). From k_e the in vitro half-life $t_{1/2}$ was calculated ($t_{1/2}=\ln(2)/k_e$).

Metabolite Profile of the [^{14}C]-Test Item

Using RAD chromatograms, the formation of metabolites (expressed as % of total radioactivity eluted in each sample) was evaluated.

II. Results and Discussion

A. Results

The mean radiochemical purity of [phenyl-UL-^{14}C]-fluopicolide was determined to be 100% and a peak retention time of 19.0-19.1 minutes was determined which was comparable to the unlabeled fluopicolide, thus confirming the identity of the radiolabelled material.

The microsomal batches of mixed-gender New Zealand White rabbit metabolised phenacetin. The remaining percentage of phenacetin was 32% in the rabbit liver microsomes after 120-minutes' incubation, thus confirming that the batches used were metabolically active.

The results from the 1 µM and 10 µM microsomal incubations are presented below. The 1 µM incubations were used for evaluation of metabolic stability and intrinsic clearance calculations, whilst the 10 µM incubations are presented for comparison purposes, indicating stability at higher concentrations.

The average extent of conversion of [phenyl-UL-^{14}C]-fluopicolide after 120 minutes' incubation was 90% and the calculated *in vitro* $t_{1/2}$ value was 36 minutes.

The recovery of radioactivity in the microsomal samples after pre-treatment was ≥94%; although the recovery was <95%, it is considered that no metabolites >5% have been missed, owing to the increased solubility and extractability of the metabolites in comparison with the parent. Therefore, a recovery of >85% is considered acceptable.

Table 5.1.1- 88: Mean recovery of radioactivity in mouse, rat, dog, and human microsomal incubation samples with [phenyl-UL-^{14}C]-fluopicolide after sample pre-treatment

Species	Time-point (min)	[phenyl-UL-^{14}C]-fluopicolide	
		1 µM	10 µM
Rabbit	1	103	95.1
	60	106	95.6
	120	93.8	88.2

All microsomal incubation samples were analysed by LC-PDA-RAD. [phenyl-UL-^{14}C]-fluopicolide was metabolised into 10 metabolites in the rabbit liver microsomal incubations from the different species. After 120 minutes of incubation, [phenyl-UL-^{14}C]-fluopicolide was on average 13.9% as total radioactivity in the chromatograms.

A total of 10 metabolites were found in the 10 µM incubations, 4 of which represented more than 5% of the total radioactivity (M2, M7, M9 and M10). The metabolites designated M1, M3, M5 and M6 are unique to the rabbit.

The observed metabolites are summarised in the table below.

Table 5.1.1- 89: Summary of metabolites of [phenyl-UL-^{14}C]-fluopicolide with rabbit liver microsomal incubations

Retention time	RRT*	Metabolite code	Metabolite code in previous study*	Replicate	% total radioactivity of the chromatogram (t=120-minutes
					New Zealand White rabbit
16.6	0.46	M1	M1	1	0.02
				2	-
19.5-19.6	0.54	M2		1	9.24
				2	8.32
23.6	0.65	M3	-	1	0.54
				2	-
24.4	0.67	M4	M2	1	0.53
				2	-
24.7-24.9	0.68	M5	-	1	0.56
				2	0.38

Retention time	RRT*	Metabolite code	Metabolite code in previous study*	Replicate	% total radioactivity of the chromatogram (t=120-minutes
					New Zealand White rabbit
25.9	0.71	M6	-	1	0.22
				2	
29.6-29.7	0.81-0.82	M7	M3	1	12.19
				2	12.90
32.7-32.8	0.90	M8	M4	1	0.60
				2	0.32
33.7-33.8	0.93	M9	M5	1	37.19
				2	39.66
34.9-35.0	0.96	M10	M6	1	23.27
				2	26.18
36.3-36.4	1	T1	T1	1	15.65
				2	12.24

*[redacted] 2019; M-653630-02-1

III. Conclusions

[Phenyl-UL-^{14}C]-fluopicolide was metabolised in mixed gender New Zealand White rabbit microsomal incubations; the average extent of conversion was 90% after 120 minutes' incubation and the calculated in vitro $t_{1/2}$ value was 36 minutes. The metabolic activity of the microsomal bath was confirmed by measuring CYP-dependent enzymatic activity (phenacetin metabolism).

A total of 10 metabolites were found in the 10 µM incubation, of which 4 represented more than 5% of the total radioactivity in at least one species (M2, M7, M9 and M10, referred to as M1, M3, M5 and M6 in the previous metabolic stability study). Four metabolites (M1, M3, M5 and M6) were not seen in the previous study in mouse, rat, dog or human microsomes and are therefore unique to the rabbit.

Assessment and conclusion by applicant:

An acceptable study yielding valid results.

Data Point:	KCA 5.1.1/20
Report Author:	[redacted]
Report Year:	2020
Report Title:	In vitro metabolism of [piridyl-2,6-14C] fluopicolide using mouse, rat, rabbit, dog and human liver microsomes
Report No:	20224452
Document No:	M-689269-01-1
Guideline(s) followed in study:	EU Commission Regulation (EC) No. 283/2013 (March 2013)
Deviations from current test guideline:	None
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

The objective of this study was to determine the metabolic stability and metabolite profiles of [pyridyl-2,6-^{14}C] fluopicolide in liver microsomes from mixed-gender CD1 mice, Wistar rats, Beagle dogs, New Zealand white rabbits and humans.

The [^{14}C]-test item was incubated at 1 and 10 µM. The incubation with 1 µM was used for metabolic stability calculations and the incubation with 10 µM was used for metabolite profiling purposes. Incubations with [^{14}C]-test item were performed for 1, 60 and 120 (±1) minutes in duplicate for all species.

An LC-PDA-RAD method was used for the analysis of incubated samples. The method was found to be adequate and the chemical stability of the test item under the conditions used was determined.

The metabolic activity of the liver microsomal batch was confirmed by measuring one CYP-dependent enzymatic activity (phenacetin metabolism).

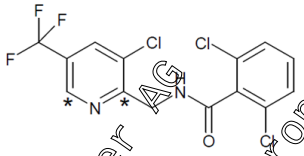
The [^{14}C]-test item showed significant metabolism in all species. The extent of conversion of the [^{14}C]-test item was 59% in dog, 92% in mouse, 93% in rabbit, 72% in human and 67% in rat liver microsomes after 120 minutes of incubation. The calculated *in vitro* $t_{1/2}$ and intrinsic clearance $CL'_{int, in\ vitro}$ values were 33 & 38, 74 & 73, 94 & 15, 31 & 134, 66 & 8 in mouse, rat, dog, rabbit and human, respectively.

A total of 16 metabolites were found in the 10µM incubation, of which M6, M9, M10, M13 and M14 represented more than 5% of the total radioactivity. No human specific metabolites were detected.

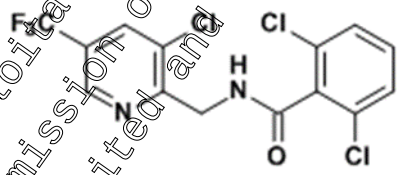
I. Materials and Methods

A. Materials

1. Test material

Radiolabelled test material	[pyridyl-2,6-14C] Fluopicolide
Chemical structure	 * *denotes position of label*
Lot number	GAR2034/4
Radiochemical purity	> 99%
Chemical purity	>99%
Specific activity	6.77 MBq/mg

2. Reference item

IUPAC Name	2,6-dichloro-N-[[3-chloro-5-(trifluoromethyl)-2-pyridinyl]methyl]-benzamide
CAS Name	benzamide, 2,6-dichloro-N-[[3-chloro-5-(trifluoromethyl)-2-pyridinyl]methyl]
Code name	AE C638206
Common name	Fluopicolide
Empirical formula	$C_{14}H_8Cl_3F_3N_2O$
CAS Number	239110-15-7
Molar mass	383.6 g/mol
Chemical structure	
Lot number	2016-012208

3. Test system

Test system	Liver microsomes form mixed gender CD1 mouse, Wistar rat, Beagle dog, New Zealand white rabbit and human
Rationale	Liver microsomes have Cytochrome P450 (Phase I) activities.
Source	[redacted]
Batch/Lot	CD-1 mouse: LGR (male) + MDO (female) Wistar rat: NNN (male) + VRM (female) New Zealand White rabbit: HTO (male) + LOB (female) Beagle dog: HSN (male) + GOV (female) Human: SNB (mixed-gender)
Storage	The liver microsomes were stored in the ultra-low freezer (≤ -75°C).

B. Study design and methods

1. Experimental phase

January 29 to March 04, 2020

2. Experimental procedures

Preparation of stock solutions

Stock solutions of the supplied [pyridyl-2,6-14C] fluopicolide were prepared in acetonitrile (ACN), the activity of the stock solutions was determined by liquid scintillation counting (LSC) and a final activity of 12.83 MBq/mL was achieved. Incubation mixtures were prepared at a final concentration 1 and 10 mM.

Pre-experiments

The LC-PDA-RAD method was optimised, by performing pilot incubation experiments with human liver microsomes. Additionally, the linearity and chemical stability of the test item under incubation conditions was confirmed. Adequate recovery efficiencies (>85%) were achieved.

Main study

For each species duplicate incubations were performed with [pyridyl-2,6-14C] fluopicolide (1 and 10 µM final concentration) in incubation buffer containing liver microsomes at different time-points. Phenacetin (30 µM final concentration) in incubation buffer containing liver microsomes at two different time-points acted as the phase I metabolic activity control (control 1). All incubations were subject to LC-PDA-RAD analysis. Samples containing 1 µM were used for metabolic stability calculations and samples containing 10 µM of test item were used for metabolic profiling purposes.

Radioactivity in all samples was determined by liquid scintillation counting (LSC).

Microsomal incubations

Duplicate incubation mixtures with [14C]-test item (1 and 10 µM final concentration) were prepared in glass vials closed with a screw cap by mixing as follows:

(1) 990 µL of 0.1 M potassium phosphate buffer pH 7.4

(2) 400 µL 2.5 mg/mL liver microsomes in 0.1 M potassium phosphate buffer pH 7.4

(3) 400 µL 5 mM NADPH in 0.1 M potassium phosphate buffer pH 7.4

(4) 200 µL 100 mM G6P with 100 U/mL G6P-DH in 0.1 M potassium phosphate buffer pH 7.4

After pre-incubation for 5±1 minutes at 37±1°C in a water bath, 10 µL of the [14C] test item Spiking 200 or Spiking 2000 solution was added resulting in final [14C] test item concentrations of 1 µM and 10 µM, respectively, in a 0.5% (v/v) aqueous solution of ACN.

Each incubation mixture was divided into three vials of 400 µL each and, from the remaining mixtures, an aliquot of 20 µL per sample was transferred to a scintillation vial and counted by LSC, after addition of 10 mL Ultima Gold cocktail.

The vials were incubated in a shaking water bath at 37±1°C. After 1±1, 60±1 and 120±1 minutes of incubation, vials were cooled down on ice, and 200 µL ACN was added. After vortex-mixing, the samples were centrifuged for 5 minutes at 16,000 g. Of each supernatant, 20 µL was transferred to scintillation vials containing 10 mL Ultima Gold cocktail and counted by LSC to determine the radioactivity in the supernatant. The rest of the supernatant was transferred to a vial and subjected to LC-PDA-RAD analysis. Samples were stored in the freezer (≤ -15°C) after analysis. The remaining pellets were stored in the freezer (≤ 15°C).

Metabolic activity controls

The metabolic competence of each of the microsomal preparations was assessed in duplicate, in parallel with the [14C]-test item incubations, by measuring the rate of phenacetin (30 µM) metabolism.

Metabolic competence in liver microsomes was determined in duplicate by incubating:

(1) 295 µL 0.1 M potassium phosphate buffer pH 7.4

(2) 50 µL 2.5 mg/mL liver microsomes in 0.1 M potassium phosphate buffer pH 7.4

(3) 100 µL 5 mM NADPH in 0.1 M potassium phosphate buffer pH 7.4

(4) 50 µL 100 mM G6P with 100 U/mL G6P-DH in 0.1 M potassium phosphate buffer pH 7.4

After pre-incubation for 5 minutes at 37±1°C, 5 µL of 3 mM phenacetin was added. After 1±1, 60 ±1 and 120 ±1 minutes of incubation, 100 µL was withdrawn and diluted with 100 µL of incubation buffer. The metabolic activity samples were stored in the freezer (≤ 15°C) for a maximum of 1 month. Prior to analysis the samples were thawed, centrifuged for 5 minutes at 16,000 g and subjected to UPLC PDA MS analysis.

Phenacetin (enzyme activity: phenacetin O-deethylase) was used as positive control for CYP1A2 activity (=Phase I metabolism).

3. Analysis

Metabolic activity

The metabolic activity of the liver microsomes was evaluated by determining the formation of detectable amounts of acetaminophen from phenacetin and by determining the disappearance of phenacetin expressed as percentage of the start concentration, based on peak area and conversion rate.

Metabolic Stability of the [^{14}C]-Test Item

The percentage [pyridyl-2,6-14C] fluopicolide remaining was defined as the ratio of the [^{14}C]-test item peak area at a specific time point and the peak area in the corresponding incubation samples at t=1 minute multiplied by 100%.

The metabolic stability was evaluated from the 1 µM incubations by plotting the natural logarithm of the percentage [^{14}C]-test item remaining versus time and performing linear regression. From the slope the elimination constant k_e was calculated (k_e=-slope). From k_e the in vitro half-life $t_{1/2}$ was calculated ($t_{1/2}=\ln(2)/k_e$). Metabolic stability was compared across species.

Metabolite Profile of the [^{14}C]-Test Item

Using RAD chromatograms, the formation of metabolites (expressed as % of total radioactivity eluted in each sample) was evaluated.

II. Results and Discussion

A. Results

The mean radiochemical purity of [pyridyl-2,6-14C] fluopicolide was determined to be 99.7% and a peak retention time of 19.1 minutes was determined which was comparable to the unlabelled fluopicolide, thus confirming the identity of the radiolabelled material.

The recovery of [pyridyl-2,6-14C] fluopicolide from the incubation media was determined to be ≥9 1% using LC-RAD for the 1 µM samples, as compared with the spiking solutions used to spike the incubation samples, which was >85% and considered acceptable.

It was determined that [pyridyl-2,6-14C] fluopicolide was stable under the microsomal conditions used as the peak area did not decrease after incubations for 120 minutes at 37˚C. Furthermore, the recovery of radioactivity of the chemical stability samples was 91.6-119%.

The microsomal batches of all species metabolised phenacetin, thus confirming that all batches were metabolically active.

The results from the 1 µM and 10 µM microsomal incubations are presented below. The 1 µM incubations were used for evaluation of metabolic stability and intrinsic clearance calculations, whilst the 10 µM incubations are presented for comparison purposes, indicating stability at higher concentrations.

The average extent of conversion of [pyridyl-2,6-14C] fluopicolide after 120 minutes' incubation was 93%, 92%, 72%, 67% and 59% in rabbit, mouse, human , rat and dog liver microsomes, respectively. The calculated *in vitro* $t_{1/2}$ values were 31, 33, 66, 74 and 94 minutes in the same respective incubations. However, as the R^2 of the metabolic stability curve for the dog liver was relatively low (0.811 and 0.760) the *in vitro* $t_{1/2}$ and intrinsic clearance $CL'_{int, in\ vitro}$ values for the dog were also calculated using two time points. The calculated *in vitro* $t_{1/2}$ value in dog liver microsomal incubations when using two time points (1 and 60 minutes) was 49 minutes.

The recovery of radioactivity in the microsomal samples after pre-treatment was ≥91% in all species.

Table 5.1.1- 90: Mean recovery of radioactivity in mouse, rat, dog, rabbit and human microsomal incubation samples with [pyridyl-2,6-14C] fluopicolide after sample pre-treatment

Species	Time-point (min)	[pyridyl-2,6-14C] fluopicolide	
		1 µM	10 µM
Mouse	1	95.5	98.2
	60	96.5	97.0
	120	91.4	95.1
Rat	1	102	109
	60	101	109
	120	101	109
Dog	1	104	102
	60	106	97.9
	120	96.5	94.4
Rabbit	1	108	100
	60	105	95.5
	120	101	93.4
Human	1	101	101
	60	97.4	95.7
	120	98.1	95.1

All microsomal incubation samples were analysed by LC-PDA-RAD. [pyridyl-2,6-14C] fluopicolide was metabolised into 8-14 metabolites in the liver microsomal incubations from the different species. After 120 minutes of incubation, [pyridyl-2,6-14C] fluopicolide was on average 3.6%, 10.9%, 35.5%, 44% and 64.3% as total radioactivity in the chromatogram in the dog, rabbit, mouse, human and rat incubations samples, respectively.

A total of 16 metabolites were found in the different incubations, 5 of which represented more than 5% of the total radioactivity (M6, M9, M10, M13 and M14). There were no human specific metabolites.

The observed metabolites in the different species are summarised in the table below.

Table 5.1.1- 91: Summary of metabolites of [phenyl-UL-^{14}C]-fluopicolide with liver microsomal incubations of different species

Retention Time (minutes)	RRT[a)]	Metabolite Code	Metabolite Code in CRL Study 20157742	Replicate	% of Total Radioactivity of the Chromatogram (t=120 minutes)				
					Mouse	Rat	Rabbit	Dog	Human
19.2	0.53	M1	M1	1	0.36	-	-	-	-
				2	-	-	-	-	-
22.0-22.1	0.60-0.61	M2		1	0.44	-	0.34	-	-
				2	0.47	-	-	-	-
22.5-22.8	0.62-0.63	M3		1	-	-	-	-	-
				2	0.41	-	-	-	-
22.9-23.8	0.63-0.66	M4		1	0.58	0.54	-	0.41	-
				2	0.64	0.49		0.54	-
23.8-24.4	0.65-0.67	M5	M2	1	0.87	0.43	0.81	1.08	0.60
				2	0.72	0.36	0.58	1.72	0.54
24.5-25.1	0.67-0.69	M6		1	**14.40**	**12.35**	1.51	0.95	0.78
				2	**14.50**	**11.10**	1.50	0.63	0.90
25.2	0.69	M7		1	-	-		-	-
				2	-	-	0.48	-	-
25.3-26.0	0.70-0.71	M8		1	0.63	-	0.61	0.64	-
				2	0.48	0.25	0.51	0.97	-
28.2-28.5	0.78	M9		1	**12.27**	**9.92**	**7.01**	2.43	**14.70**
				2	**12.34**	**9.11**	**7.17**	3.19	**13.91**
29.3-29.6	0.81	M10	M3	1	**10.12**	0.79	**12.96**	**33.75**	**22.00**
				2	**9.91**	0.82	**12.82**	**33.76**	**20.82**
30.9-31.0	0.85	M11		1	-	-	0.39	-	-
				2	-	-	0.31	-	-
32.4-32.9	0.89-0.90	M12	M4	1	0.34	-	0.43	0.90	0.32
				2		-	0.51	0.82	0.31
33.6-33.8	0.93	M13	M5	1	**11.84**	3.24	**37.90**	**17.94**	**7.35**
				2	**11.48**	3.20	**37.52**	**7.15**	**7.76**
34.7-35.0	0.95-0.96	M14	M6	1	**14.84**	**8.88**	**26.92**	**37.52**	**10.70**
				2	**14.75**	**8.56**	**27.45**	**47.12**	**9.86**
35.6	0.98	M15	M7	1	0.36[c)]	-	-	0.21[b)]	-
				2	-	-	-	-	-
36.2-36.5	1	TI		1	**32.97**	**63.24**	**10.80**	3.86	**43.22**
				2	**34.05**	**65.35**	**10.96**	3.34	**45.51**
37.2-37.5	1.02-1.03	M16	M8	1	0.33	0.60	0.31	0.51	0.34
				2	0.25	0.73	0.19	0.75	0.40

-: not present; **Bold** values indicate containing ≥5% of total radioactivity; TI: [^{14}C]-test item
[a)] RRT: Relative retention time compared to parent compound
[b)] detected in t = 1 minutes sample
[c)] detected in t = 60 minutes sample

III. Conclusions

[Pyridyl-2,6-14C] fluopicolide was metabolised in CD1 mice, Wistar rat, Beagle dog, New Zealand White rabbit and human liver microsomal incubations; the extent of conversion was 93%, 92%, 72%, 67% and 59% in rabbit, mouse, human , rat and dog liver microsomes, respectively. The calculated *in vitro* $t_{1/2}$ values were 31, 33, 66, 74 and 94 minutes in the same respective incubations A total of 16 metabolites were found, of which 5 represented more than 5% of the total radioactivity in at least one species (M6, M9, M10, M13 and M14).

No human specific metabolites were detected.

Assessment and conclusion by applicant:
An acceptable study yielding valid results.

Data Point:	KCA 5.1.1/21
Report Author:	[redacted]
Report Year:	2020
Report Title:	[Pyridyl-2,6-14C]fluopicolide - Bile excretion in male rats
Report No:	EnSa-20-0204
Document No:	M-681576-01-1
Guideline(s) followed in study:	Regulation (EC) No 1107/2009 of the European Parliament and of the Council amended by Commission Regulation (EU) No 283/2013. OECD Guideline for the Testing of Chemicals No. 417 Toxicokinetics, July 22, 2010 US-EPA OCSPP 870.7485 Japanese MAFF Test Guideline 12 Nousan 8147
Deviations from current test guideline:	None
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary

Absorption, metabolism, and excretion of [pyridyl-2,6-^{14}C]-fluopicolide were investigated in a group of 6 bile duct-cannulated male rats, which received a low dose of 2 mg/kg bw by oral gavage as an aqueous suspension in 0.5% Tragacanth. Over a period of 48 h after dosing, total radioactivity related to the parent and its metabolites was determined for urine, faeces, and bile, and in the carcass, skin, and gastro-intestinal tract (GIT).

Of the administered radioactivity, a total of 105.82% was recovered. The proportions of the total radioactive dose recovered were as follows:

	Percent dose administered	CV		Percent dose recovered	CV
Bile	99.59	1.2		94.11	0.5
Faeces	1.51	8.7		1.42	9.0
Urine	4.17	10.1		3.94	9.8
Total excreted	**105.26**	1.1		**99.47**	0.1
Body excluding GIT	0.546	9.1		0.516	8.3
GIT	0.019	69.8		0.018	69.4
Total in body	**0.565**	10.6		**0.533**	9.9
Balance	**105.82**	1.1	**Normalization factor**	0.945	1.2
			Absorption (%)	**98.56**	0.1

Of the recovered dose, 94.1% was detected in the bile. Absorption was calculated as 98.56%.

Excretion was almost complete at 24 h at which time >99% of the recovered dose had been excreted in urine, bile and faeces. Excretion was predominantly biliary.

The excretion and the metabolic behaviour of [pyridyl-2,6-^{14}C]-fluopicolide in bile duct-cannulated male rats were characterized as follows:

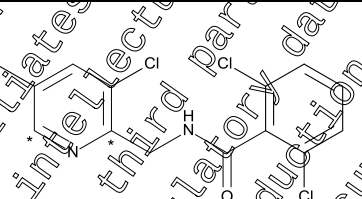
- Fluopicolide showed a high absorption rate in male rats following oral administration of 2 mg/kg bw, amounting to over 98%.
- Absorbed radioactivity was quickly and efficiently eliminated from the body and was nearly complete at sacrifice 48 h after dosing, the main portion of radioactivity being eliminated by 24 h.
- Excretion of radioactivity was predominantly biliary (>94%). Only 1.42% of the recovered dose was detected in faeces and 3.9% in urine. Only 0.52% remained in the carcass, 0.02% in the GIT.
- Seven metabolites were identified in bile and urine of male rats, of which HO812104 and HO812103 (cysteine and cysteine-glycine conjugates of AE C643890 (3-OH 206)) were major metabolites (53% and 20% respectively in bile, ≤0.06% in urine).
- Minor metabolites were HO812107, HO812106, HO812105 and AE C643890 (3-OH 206). PCA was detected as a minor metabolite in urine only. Parent compound was not detected in urine or bile.
- The most important metabolic route was hydroxylation of the phenyl ring leading to AE C643890 (3-OH 206) followed by cysteine and cysteine-glycine conjugation (via glutathione) in position 3 of the phenyl ring.
- The glucuronic acid conjugate of AE C643890 (3-OH 206) was also observed. The position of the conjugation was determined as 3-OH of the phenyl ring after enzymatic cleavage of the conjugate.
- Conjugation of AE C643890 (3-OH 206) with cysteine (via glutathione) was observed at position 2 or position 4 of the phenyl ring. The conjugate of AE C643890 (3-OH 206) with cysteine-glycine (via glutathione) in position 2 of the phenyl ring was also detected.
- Cleavage of the molecule at the amide bond, and conjugation with cysteine (via glutathione) of the cleaved molecule, were observed as minor reactions.

The results were in good agreement with the results of the companion study in male rats with [phenyl-UL-^{14}C]-fluopicolide. Based on these results, the metabolic behaviour of [pyridyl-2,6-^{14}C]-fluopicolide in male rats is well understood.

I. Materials and methods

A. Materials

1. Test material

IUPAC Name	2,6-dichloro-N-[(3-chloro-5-trifluoromethyl-2-pyridyl)methyl]benzamide
CAS name	Benzamide, 2,6-dichloro-N-([3-chloro-5-(trifluoromethyl)-2-pyridynil]methyl) (9Cl)
CAS number	239110-15-7
Code name	AE C638206 or BCS-AM59797
Common name	Pyridine carboxylic acid (PCA)
Empirical formula	$C_{14}H_8Cl_2F_3N_2O$
Molar mass	383.6 g/mol
Radiolabelled test material	[Pyridyl-2,6-^{14}C]-fluopicolide
Chemical structure	 ** denotes positions of label*
Lot number	KML 10553
Radiochemical purity	>99% (HPLC, radio- and UV detection)
Specific radioactivity	6.77 MBq/mg, 182.97 µCi/mg = 406200 dpm/µg
Storage	At ≤-18°C

2. Vehicle: Aqueous 0.5% Tragacanth

3. Test animals:

Species: Rat
Strain: Wistar Unilever Crl:(Wi)WU BR
Age: Approximately 7 weeks on delivery
Acclimatization: One week
Weight at dosing: 184-219 g
Source: [redacted]
Identification: Tail marking
Diet: Rodent maintenance diet V1534-000 10 mm or V1530-000 powdered (ssniff Spezialdiäten GmbH, Soest, Germany)
Water: Provided *ad libitum* (tap water from municipal supply)
Housing: Makrolon cages with wood shavings, then Makrolon metabolism cages after dosing
Environmental conditions
  Temperature: 22 ± 2˚C
  Humidity: 20-39%
  Photoperiod: 12 hours
  Air changes Air-conditioned; changes not stated

B. Study design and methods

1. In life dates: 09 January to 14 January 2020

2. Dose regimen and design of test

Administration		Animals		Duration	Description
Dose	Route	No.	Sex		
2 mg/kg bw	p.o.	6	Male	48 h	Single low dose after bile-duct cannulation

The dose level was selected to represent a non-toxic dose high enough to allow for metabolite identification. The ADI of fluopicolide was based on the NOAEL of 7.9 mg/kg bw/d from a 78 week mouse study, supported by a 2-year rat study (NOAEL of 8.4 mg/kg bw/d). Therefore, the selected dose of 2 mg/kg bw was considered appropriate.

3. Preparation of dosing formulation

The radiolabelled test material (KML 10553) it was dissolved in acetonitrile and stored at approximately -18 C. The purity of the test item in the stock solution was confirmed by HPLC.

The dosing suspension was prepared by first concentrating the stock solution to near dryness under a nitrogen stream, then formulating in 0.5% aqueous Tragacanth using an ultrasonic bath. The suspension was then placed on a magnetic stirrer overnight at approximately 5°C and then at room temperature during actual dosing.

After dosing, the radiochemical purity of the suspension was confirmed by HPLC analysis, while the amount of radioactivity was determined by LSC.

4. Dose administration

The concentration of the dosing suspension was calculated to achieve an administered amount of approximately 2 mg test material/kg bw, assuming an animal weight of 200 g.

The suspension was administered by oral gavage, using a feeding cannula. The amount and resulting radioactivity administered to each rat is shown below.

Administered per rat					
Fluo-picolide [mg]	Volume [mL]	Radioactivity [dpm]	Nominal dose [mg/kg bw]	Actual dose [mg/kg bw]	Mean rat weight [g]
0.39	1.0	159,794,064	2	1.95	202

5. Collection of samples

Urine, bile, and faeces samples were collected at various times separately for each animal. Skin, carcass and gastro-intestinal tract (GIT) were dissected from the animals at sacrifice and weighed. The collection intervals for the respective samples are shown below.

Time of collection after dosing (h)			
Urine	Faeces	Bile	GIT
0-4, 4-8, 8-24, 24-48	0-24, 24-48	0-4, 4-8, 8-24, 24-48	48

The animals were sacrificed using an overdose of pentobarbital-sodium.

6. Preparation and analysis of samples

The funnels for urine collection were rinsed into the corresponding urine fraction, then weighed prior to analysis. For bile, aliquots were taken for analysis. Faecal fractions were diluted with water, weighed, then homogenized and combusted prior to analysis. Skin, carcass and GIT were homogenized prior to taking aliquots for combustion and analysis.

Measurement of radioactivity in the liquid samples was carried out by LSC. All solid samples were combusted in an oxygen atmosphere using an oxidiser. The released $^{14}CO_2$ was absorbed in a special alkaline reagent. After mixing the reagent with liquid scintillator the radioactivity was determined by LSC:

For liquids, the volume of the entire sample was determined and aliquots (1-3 replicates) were subjected to LSC. The mean measurement efficiency was 87.55%. Solid samples were weighed and combusted in oxygen, the $^{14}CO_2$ being absorbed into Oxysolv scintillator-liquid prior to LSC.

Counting times were generally from a few seconds to 20 min. Measurement was stopped after reaching a 2-sigma of 0.21% or after 20 min.

For all steps during sample preparation (extraction and clean-up), the values for individual fractions were normalized to 100% of recovered radioactivity.

For identification of parent and metabolites in the samples, the urine sample aliquots from all animals were combined into a representative pool, all sampling intervals being combined into a pool for 0-48 h. The bile samples were similarly pooled (0-48 h) for analysis. Owing to the low amount of radioactivity found in the faeces (mean <2% of recovered dose), parent and metabolites were not investigated for this matrix.

Reversed phase HPLC with an acetonitrile/buffered water gradient was used to analyse parent and metabolites in urine and bile. The metabolite fractions were isolated using an established HPLC method. Further purification was achieved by varying the column type or the solvent gradient. These same methods were used for profiling and quantification in the study with the phenyl label.

7. Treatment and evaluation of data

The data were processed and evaluated using PhaLIMS 4.7 (Pharmacokinetic-LIMS). The data were checked for outliers using an outlier test, and any such values were excluded from subsequent calculations. If more than one-third of values contributing to a mean were below the LOQ, the calculated mean was also set below LOQ and excluded from arithmetic means.

Radioactivity in percent of total dose administered was normalized to percentage of total radioactivity recovered. Concentrations represented active substance equivalents, so taking account of the parent compound and all radiolabelled metabolites, and were dose normalized.

An assumption of even distribution into the body organs was assumed, taking the animal weight at sacrifice as a measure of body volume. The percentage amounts in organs were obtained from the product of dose-normalized concentrations and the corresponding gamma-values (equivalent to percentage wet-weight contribution to the body weight).

If the mean values for fraction samples or subsets were calculated from values with <LLQ, half of the LLQ was used in place of the non-determined value.

For the HPLC analyses, all peaks corresponding to a signal approximately 2.5x background were integrated. The LOQ was set at the level of the LOD.

Mass spectroscopy (electrospray ionization) and/or NMR spectroscopy were deployed as appropriate.

Parent compound and metabolites were numbered with peak identities ID 1 to ID 25, based on retention time and spectroscopic behaviour.

II. Results and Discussion

1. Stock solution and dosing suspension

Purity of the test material in the stock solution was proven by HPLC, identity by LC-MS and LC-MS/MS. Radiochemical purity of the dosing suspension was checked by HPLC and found to be 99.94%.

The actual dose delivered was 1.95 mg/kg bw (nominal 2 mg/kg bw).

2. Balance of radioactivity

105.82% of the administered dose was recovered in the test.

Table 5.1.1-92: Balance of radioactivity in excreta and tissue of rats following oral [pyridyl-2,6-^{14}C]-fluopicolide at nominal 2 mg/kg bw. Mean and CV (coefficient of variation)

	Percent dose administered	CV		Percent dose recovered	CV
Bile	99.59	1.2		94.11	0.1
Faeces	1.51	8.7		1.42	9.0
Urine	4.17	10.1		3.94	9.8
Total excreted	**105.26**	1.1		**99.47**	0.1
Body excluding GIT	0.546	9.1		0.516	8.3
GIT	0.019	69.8		0.018	69.4
Total in body	**0.565**	10.6		**0.533**	9.9
Balance	**105.82**	1.1	**Normalization factor**	0.945	1.2
			Absorption (%)	**98.56**	0.1

3. Absorption

In the time period from 0 (dosing) to 48 h post-dose the total absorbed was 98.6% of recovered dose (total in urine, bile and body without the GIT), while 94.1% was detected in the bile.

4. Excretion

The major part of the radioactivity was detected in the bile. At 24 h after administration, more than 99% of the recovered dose had been excreted in bile, faeces, and urine (approximately 4% in urine, 1.4% in the faeces).

Table 5.1.1- 93: Excretion of radioactivity (% of dose administered and recovered), Mean and coefficient of variation (CV) after oral 2 mg/kg bw

Time (h post dose)	% of dose administered	CV	% of dose recovered	CV
Bile				
4	59.45	15.8	56.14	15.3
8	29.64	20.2	28.04	20.9
24	10.22	33.3	9.66	33.5
48	0.28	31.2	0.26	32.4
Faeces				
24	1.44	7.0	1.36	7.1
48	0.07	61.9	0.06	62.8
Urine				
4	0.95[a]	100.4[a]	0.89[a]	100.2[a]
8	1.92	68.7	1.82	69.2
24	1.23	106.3	1.15	105.9
48	0.07	67.1	0.07	66.4
Sum excreted:	**105.26**	1.1	**99.47**	0.1
Normalization factor			0.945	1.2

a Mean value calculated with half LLOQ

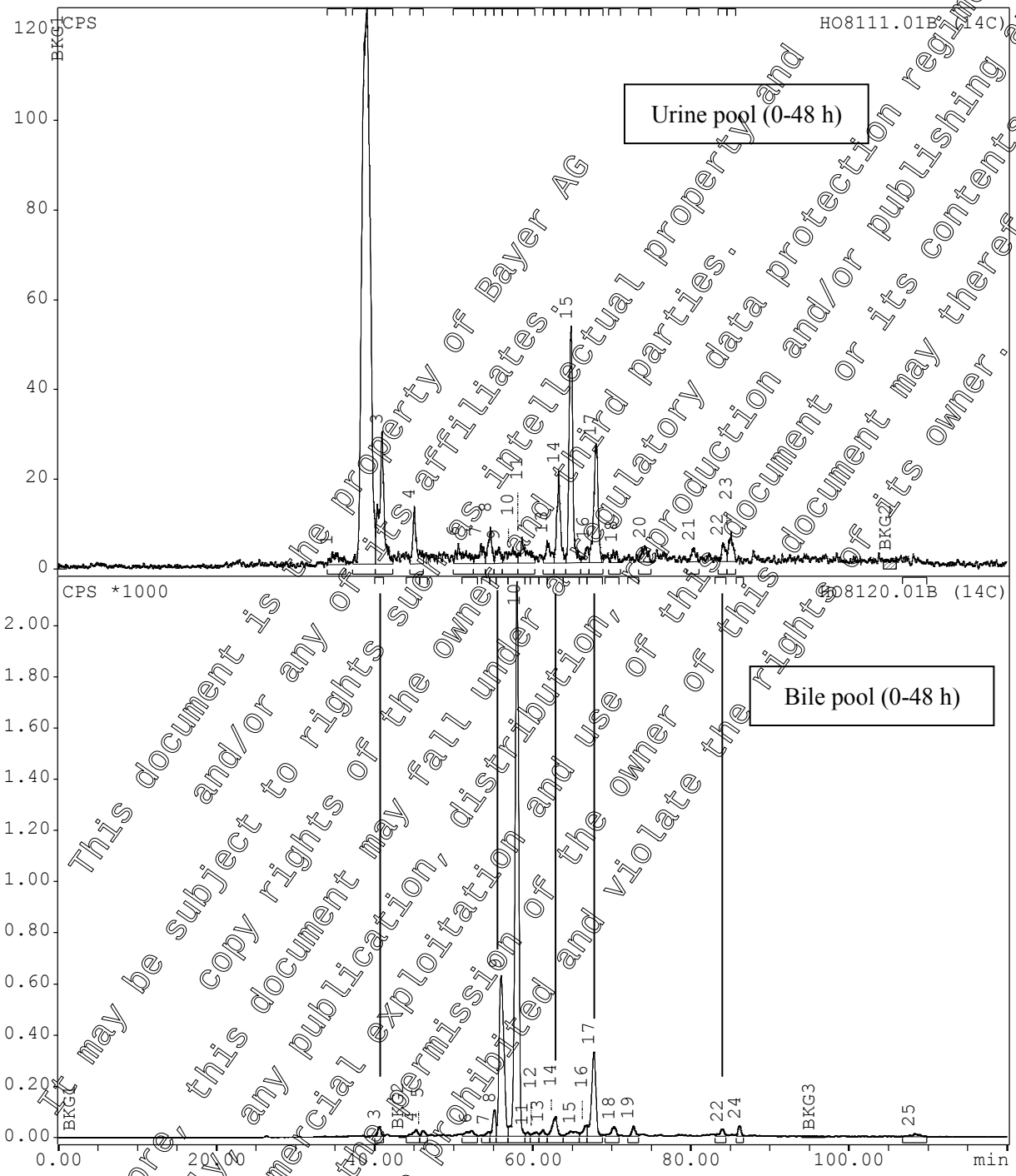
5. Residues in the carcass and GIT

At sacrifice, only low levels of radioactivity were found in the bodies excluding GIT (0.516% of the recovered dose). Only 0.018% of radioactivity was detected in the GIT.

6. Metabolism

Metabolite profiles showed extensive metabolic transformation. The profile for bile and urine is shown below. Parent compound was not detected in either urine or bile. Faecal samples were not analysed, owing to the low amount of radioactivity detected in that matrix.

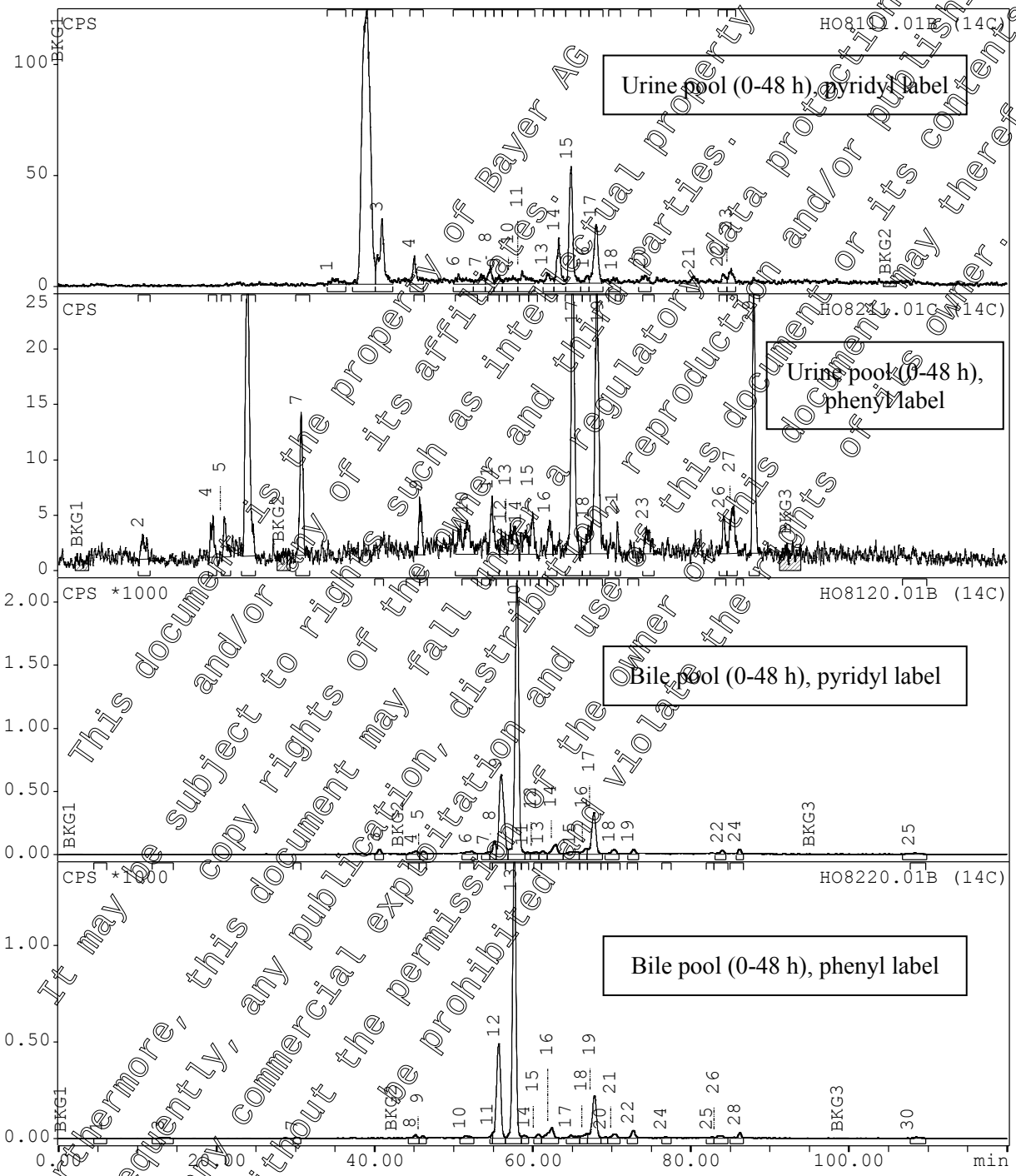
Figure 5.1.1-20: Comparison of ^{14}C-chromatograms of urine and bile from male rats



Note: Metabolites with the same identified chemical structure in urine and bile are shown bonded with drawn vertical lines

There were label-specific differences between urine samples from the present study and from the companion study with the phenyl label (see also next study summary). The bile profile was comparable between the two studies. A comparison is shown below.

Figure 5.1.1-21: Comparison of ^{14}C-chromatograms of urine and bile from male rats



The peak IDs (based on retention times) and identification of the metabolites from the pyridyl label is shown in the following table:

Table 5.1.1- 94: Overview of identified and characterized metabolites in urine and bile of male rats (pyridyl label) after oral 2 mg/kg bw

Peak ID	Appearance in		Report name
	urine	bile	
1	X	---	unknown 1
2	X	---	PCA (AE C657188)
3	X	X	HO812107
4	X	X	unknown 2
5	---	X	unknown 3
6	X	X	unknown 4
7	X	X	unknown 5
8	X	X	unknown 6
9	X	X	HO812103
10	X	X	HO812104
11	X	X	unknown 7
12	---	X	unknown 8
13	X	X	unknown 9
14	X	X	HO812106
15	X	X	unknown 10
16	X	X	unknown 11
17	X	X	HO812105
18	X	X	unknown 12
19	---	X	unknown 13
20	X	---	unknown 14
21	X	---	unknown 15
22	X	X	AE C643890 (3-OH 206)
23	X	---	unknown 16
24	---	X	unknown 17
25	---	X	unknown 18

X = detected; --- = not detected

Table 5.1.1- 95: Quantitation of metabolites in urine of male rats (pyridyl label) after oral 2 mg/kg bw

Peak ID	Report name	Rt (min)	Area (%)	% of dose
1	unknown 1	34.6	1.21	0.05
2	PCA (AE C657188)	39.0	53.56	2.23
3	HO812107	40.9	7.60	0.32
4	unknown 2	45.0	2.54	0.11
6	unknown 4	50.6	1.70	0.07
7	unknown 5	53.4	1.14	0.05
8	unknown 6	54.6	1.51	0.06
9	HO812103	55.7	0.84	0.04
10	HO812104	57.5	1.34	0.06
11	unknown 7	58.7	1.92	0.08
13	unknown 9	61.8	1.21	0.05
14	HO812106	63.3	3.77	0.16
15	unknown 10	64.9	10.07	0.42
16	unknown 11	67.0	0.75	0.03
17	HO812105	68.0	6.15	0.26
18	unknown 12	70.6	0.63	0.03
20	unknown 14	74.2	1.10	0.05
21	unknown 15	80.4	0.75	0.03
22	AE C643800 (3-OH 206)	84.0	0.86	0.04
23	unknown 16	85.1	1.33	0.06
Total				4.17
Sum of identified				3.09
Sum of characterized				1.08

Table 5.1.1- 96: Quantitation of metabolites in bile of male rats (pyridyl label) after oral 2 mg/kg bw

Peak ID	Report name	Rt (min)	Area (%)	% of dose
3	HO812107	40.6	0.86	0.86
4	unknown 2	45.2	0.87	0.87
5	unknown 3	46.1	0.56	0.56
6	unknown 4	52.2	1.15	1.15
7	unknown 5	54.4	0.48	0.48
8	unknown 6	55.2	2.16	2.15
9	HO812103	56.0	19.86	19.78
10	HO812104	58.0	53.30	53.08
11	unknown 7	59.3	0.34	0.34
13	unknown 9	61.3	0.67	0.67
14	HO812106	62.9	2.67	2.66
15	unknown 10	65.3	1.24	1.23
16	unknown 11	66.8	1.25	1.24
17	HO812105	67.7	9.46	9.42
18	unknown 12	70.3	1.30	1.29
19	unknown 13	72.3	0.99	0.99
22	AE C643890 (3-OH 206)	84.0	0.90	0.90
24	unknown 17	86.2	0.85	0.85
25	unknown 18	108.3	0.52	0.52
Total				99.59
Sum of identified				86.69
Sum of characterized				12.90

Table 5.1.1- 97: Balance in male rats (pyridyl label) after oral 2 mg/kg bw, as % of dose

Peak ID	Report name	Urine (0-48 h)	Bile (0-48 h)	Total
2	PCA (AE C657188)	2.23	---	2.23
3	HO812107	0.32	0.86	1.17
9	HO812103	0.04	19.78	19.81
10	HO812104	0.06	53.08	53.14
14	HO812106	0.16	2.66	2.82
17	HO812105	0.26	9.42	9.68
22	AE C643890 (3-OH 206)	0.04	0.90	0.93
Total identified		**3.09**	**86.69**	**89.78**
1	unknown 1	0.05	---	0.05
4	unknown 2	0.11	0.86	0.97
5	unknown 3	---	0.56	0.56
6	unknown 4	0.07	1.15	1.22
7	unknown 5	0.05	0.48	0.53
8	unknown 6	0.06	2.15	2.21
11	unknown 7	0.08	0.34	0.42
12	unknown 8	---	0.57	0.57
13	unknown 9	0.05	0.67	0.72
15	unknown 10	0.42	1.23	1.65
16	unknown 11	0.03	1.24	1.28
18	unknown 12	0.03	1.29	1.32
19	unknown 13	---	0.99	0.99
20	unknown 14	0.05	---	0.05
21	unknown 15	0.03	---	0.03
23	unknown 16	0.06	---	0.06
24	unknown 17	---	0.85	0.85
25	unknown 18	---	0.52	0.52
Total characterized		**1.08**	**12.90**	**13.97**
Total				**103.75**

Two major metabolites were identified in bile as cysteine-glycine and cysteine conjugates of AE C643890 (3-OH 206): HO812103 amounted to 19.78% of the administered dose and HO812104 amounted to 53.08%. They were also detected in urine in very low amounts (≤0.06%).

HO812105 (a glucuronic acid conjugate of AE C643890) amounted to 9.42% in bile and to 0.26% in urine.

Three minor metabolites were identified in bile: HO812107, HO812106 and AE C643890 (3-OH 206), each amounting to ≤2.66% of the administered dose. They were also detected in urine, each amounting to ≤0.32%.

One metabolite was only identified in urine, PCA (AE C657188), amounting to 2.23% of administered dose.

More metabolites were indicated by broad non-resolved zones in the chromatograms. Unidentified metabolites were characterised by their extraction and chromatographic behaviour.

All results corresponded well with the findings in the accompanying ADME study with administration of [phenyl-UL-^{14}C]-fluopicolide. The metabolite pattern in the urine samples from the current study and the study with the phenyl label was similar, excepting the individual label-specific metabolites.

Elimination of the phenyl moiety led to pyridyl label specific metabolites that could not be detected in the phenyl label study. The following label-specific metabolites occurred at levels up to 2.23% of the dose administered: PCA (only detected in urine) and HO812107.

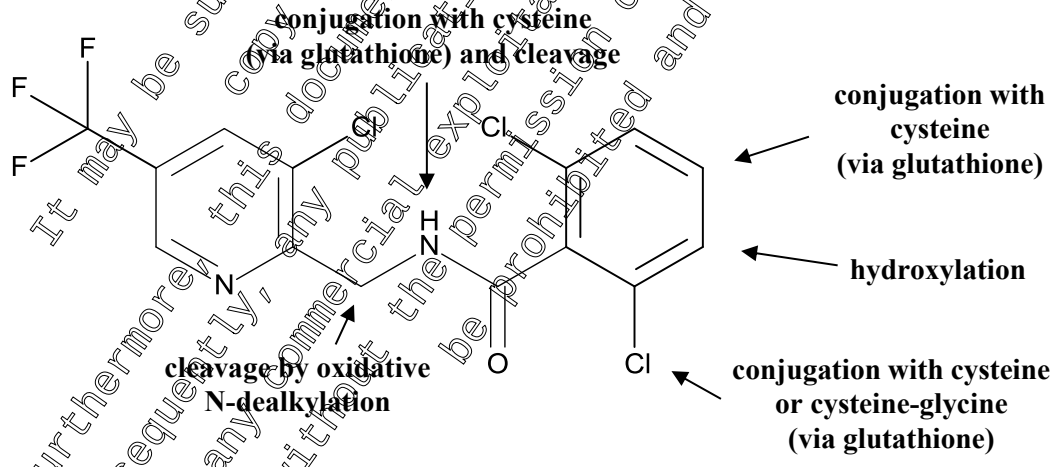
7. Metabolic pathway

In general, the main metabolic routes observed for rats treated with [pyridyl-2,6-^{14}C]-fluopicolide and [phenyl-UL-^{14}C]-fluopicolide were identical. Low levels of label-specific metabolites were identified.

The principal metabolic reactions of [pyridyl-2,6-^{14}C]-fluopicolide in the rat are listed below:

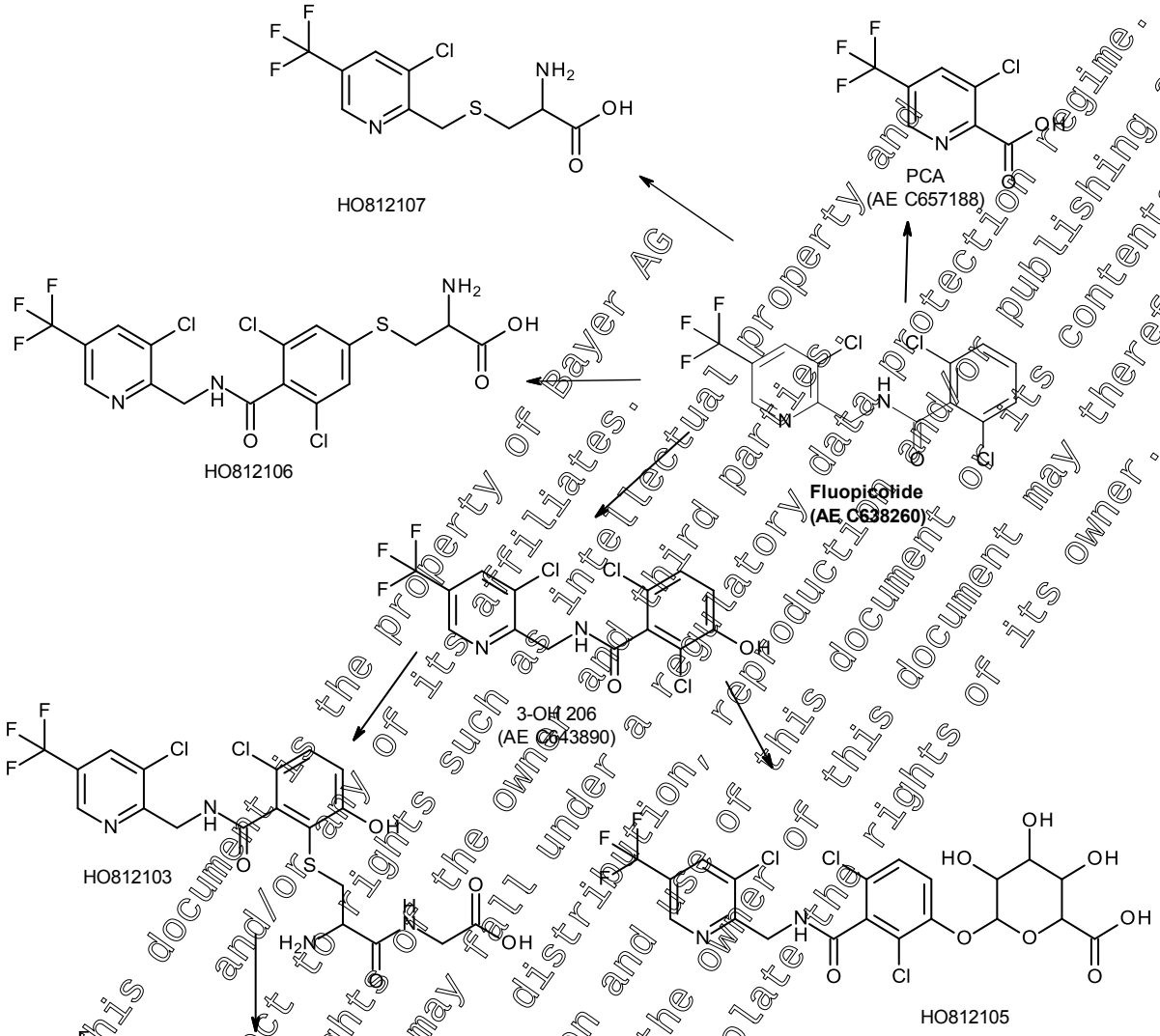
- hydroxylation in position 3 of the phenyl ring leading to AE C643890 (3-OH 206)
- subsequent conjugation with glucuronic acid in position 3 of the phenyl ring of AE C643890 (3-OH 206)
- conjugation AE C643890 (3-OH 206) in position 2 of the phenyl ring with cysteine or with cysteine-glycine (via glutathione conjugation and subsequent loss of glutamic acid)
- conjugation with cysteine (via glutathione conjugation) in position 4 of the phenyl ring of the parent molecule
- cleavage of the parent molecule at the amide bond by oxidative N-dealkylation
- conjugation with cysteine on the amide bridge linkage and subsequent cleavage of the molecule (via glutathione conjugation)

The figure below shows schematically the positions in the molecule, which are mainly involved in the metabolic reactions:



The proposed biotransformation pathway of [pyridyl-2,6-^{14}C]-fluopicolide is presented in the figure below.

Figure 5.1.1-22: Proposed metabolic pathway of [pyridyl-2,6-^{14}C]-fluopicolide in rats



III. Conclusions

The excretion and the metabolic behaviour of [pyridyl-2,6-^{14}C]-fluopicolide in bile duct-cannulated male rats were characterized as follows:

- Fluopicolide showed a high absorption rate in male rats following oral administration of 2 mg/kg bw, amounting to over 98%.
- Absorbed radioactivity was quickly and efficiently eliminated from the body and was nearly complete at sacrifice 48 h after dosing, the main portion of radioactivity being eliminated by 24 h.
- Excretion of radioactivity was predominantly biliary (>94%). Only 1.42% of the recovered dose was detected in faeces and 3.9% in urine. Only 0.52% remained in the carcass, 0.02% in the GIT.
- Seven metabolites were identified in bile and urine of male rats, of which HO812104 and HO812103 (cysteine and cysteine-glycine conjugates of AE C643890 (3-OH 206)) were major metabolites (53% and 20% respectively in bile, ≤0.06% in urine).
- Minor metabolites were HO812107, HO812106, HO812105 and AE C643890 (3-OH 206). PCA was detected as a minor metabolite in urine only. Parent compound was not detected in urine or bile.
- The most important metabolic route was hydroxylation of the phenyl ring leading to AE C643890 (3-OH 206) followed by cysteine and cysteine-glycine conjugation (via glutathione) in position 3 of the phenyl ring.
- The glucuronic acid conjugate of AE C643890 (3-OH 206) was also observed. The position of the conjugation was determined as 3-OH of the phenyl ring after enzymatic cleavage of the conjugate.
- Conjugation of AE C643890 (3-OH 206) with cysteine (via glutathione) was observed at position 2 or position 4 of the phenyl ring. The conjugate of AE C643890 (3-OH 206) with cysteine-glycine (via glutathione) in position 2 of the phenyl ring was also detected.
- Cleavage of the molecule at the amide bond, and conjugation with cysteine (via glutathione) of the cleaved molecule, were observed as minor reactions.

The results were in good agreement with the results of the companion study in male rats with [phenyl-UL-^{14}C]-fluopicolide (see following study summary). Based on these results, the metabolic behaviour of [pyridyl-2,6-^{14}C]-fluopicolide in male rats is well understood.

Assessment and conclusion by applicant:

An acceptable study yielding valid conclusions.

Data Point:	KCA 5.1.1/22
Report Author:	[redacted]
Report Year:	2020
Report Title:	[phenyl-UL-14C]fluopicolide - Bile excretion in male rats
Report No:	EnSa-19-0735
Document No:	M-681498-01-1
Guideline(s) followed in study:	Regulation (EC) No 1107/2009 of the European Parliament and of the Council amended by Commission Regulation (EU) No 283/2013 OECD Guideline for the Testing of Chemicals No. 417, Toxicokinetics, July 22, 2010 US EPA OCSPP 870.7485 Japanese MAFF Test Guideline 12 Nousan 8147
Deviations from current test guideline:	None
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities.
Acceptability/Reliability:	Yes

Executive summary

Absorption, metabolism and excretion of [phenyl-UL-^{14}C]-fluopicolide were investigated in a group of 6 bile duct-cannulated male rats, which received a low dose of 2 mg/kg bw by oral gavage as an aqueous suspension in 0.5% Tragacanth. Over a period of 48 h after dosing, total radioactivity related to the parent and its metabolites was determined for urine, faeces and bile, and in the carcass, skin and gastro-intestinal tract (GIT).

Of the administered radioactivity, a total of 101.94% was recovered. The proportions of the total radioactive dose recovered were as follows:

	Percent dose administered	CV		Percent dose recovered	CV
Bile	91.84	4.7		90.07	3.3
Faeces	3.74	58.6		3.69	59.2
Urine	4.01	30.8		3.94	31.6
Total excreted	**99.59**	2.2		**97.69**	0.3
Body excluding GIT	2.204	13.2		2.159	11.5
GIT	0.153	73.4		0.148	69.7
Total in body	**2.357**	16.2		**2.307**	14.1
Balance	**101.94**	2.5	**Normalization factor**	0.981	2.4
			Absorption (%)	**96.17**	2.2

Of the recovered dose, 90.1% was detected in the bile. Absorption was calculated as 96.17%.

Excretion was almost complete at 24 h, at which time >90% of the recovered dose had been excreted in urine, bile and faeces. Excretion was predominantly biliary.

The excretion and metabolic behaviour of [phenyl-UL-^{14}C]-fluopicolide in bile-duct cannulated male rats may be characterized as follows:

- Fluopicolide shows a high absorption rate in male rats following administration of 2 mg/kg bw. At 48 h post-dose the recovered dose amounted to about 96%.
- Excretion was predominantly via the bile, with about 92% of the administered radioactivity found in the bile and about 4% in the urine.
- At sacrifice about 2.2% of the administered radioactivity was found in the bodies excluding GIT, a low amount of radioactivity remaining in the GIT (<0.2%).
- Absorbed radioactivity was quickly and efficiently eliminated from the bodies of the rats. Elimination was nearly complete at sacrifice 48 hours after administration, by which time point >97% of the recovered dose was eliminated.
- Only small amounts of parent compound were excreted with the urine.
- Six metabolites were identified in bile and urine of male rats. The metabolites HO812104 (49.67% of the administered dose) and HO812103 (19.03%) were found as major metabolites in male rats (cysteine and cysteine-glycine conjugates of 3-OH 206).
- Minor metabolites were HO812105 (9.41%) and HO812106 (2.84%). The metabolites 3-OH 206 and BAM (2,6-dichlorobenzamide) were also identified, amounting to less than 2% of dose.
- The most important metabolic route of [phenyl-UL-^{14}C]-fluopicolide was hydroxylation of the phenyl ring leading to 3-OH 206, followed by cysteine and cysteine-glycine conjugation (via glutathione) in position 3 of the phenyl ring.
- Conjugation was the most important metabolic reaction in the elimination process of [phenyl-UL-14C]-fluopicolide: Conjugation of 3-OH 206 with cysteine, cysteine-glycine (via glutathione) and glucuronic acid, as well as conjugation of the parent compound with cysteine was observed.
- Cleavage of the amide bond by oxidative N-dealkylation was observed as a minor reaction.

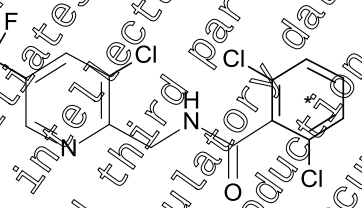
The results are in good agreement with the results of the companion study in bile duct cannulated male rats with [pyridyl-2,6-^{14}C]-fluopicolide (see preceding study summary).

Based on these results the metabolic behaviour of [phenyl-UL-^{14}C]-fluopicolide in male rats is well understood.

I. Materials and methods

A. Materials

1. Test material

IUPAC Name	2,6-dichloro-N-[(3-chloro-5-trifluoromethyl-2-pyridyl)methyl]benzamide
CAS name	Benzamide, 2,6-dichloro-N-([3-chloro-5-(trifluoromethyl)-2-pyridynil]methyl) (9Cl)
CAS number	239110-15-7
Code name	AE C638206 or BCS-AM59797
Common name	Pyridine carboxylic acid (PCA)
Empirical formula	$C_{14}H_8Cl_3F_3N_2O$
Molar mass	383.6 g/mol
Radiolabelled test material	[Phenyl-UL-^{14}C]-fluopicolide
Chemical structure	 * *denotes positions of label*
Lot number	KML 10668
Radiochemical purity	>99% (HPLC, radio- and UV detection)
Specific radioactivity	3.53 MBq/mg, 182.97 µCi/mg = 406200 dpm/µg
Storage	At ≤-18°C

2. Vehicle: Aqueous 0.5% Tragacanth

3. Test animals:

Species: Rat
Strain: Wistar Unilever Crl:(Wi)WU BR
Age: Approximately 7 weeks on delivery
Acclimatization: One week
Weight at dosing: 170-192 g
Source: [redacted]
Identification: Tail marking
Diet: Rodent maintenance diet V1534-000 10 mm or V1530-000 powdered (ssniff Spezialdiäten GmbH, Soest, Germany)
Water: Provided *ad libitum* (tap water from municipal supply)
Housing: Makrolon cages with wood shavings, then Makrolon metabolism cages after dosing

Environmental conditions
Temperature: 22 ± 2°C
Humidity: 20-39%
Photoperiod: 12 hours
Air changes: Air-conditioned; changes not stated

B. Study design and methods

1. Initiation and completion dates: 21 January 2019 to 02 April 2020

2. Dose regimen and design of test

Administration		Animals		Duration	Description
Dose	Route	No.	Sex		
2 mg/kg bw	p.o.	6	Male	48 h	Single low dose after bile-duct cannulation

The dose level was selected to represent a non-toxic dose high enough to allow for metabolite identification. The ADI of fluopicolide was based on the NOAEL of 7.9 mg/kg bw/d from a 78 week mouse study, supported by a 2-year rat study (NOAEL of 8.4 mg/kg bw/d). Therefore, the selected dose of 2 mg/kg bw was considered appropriate.

3. Preparation of dosing formulation

The radiolabelled test material (KML-10553) it was dissolved in acetonitrile and stored at approximately -70 C. The purity of the test item in the stock solution was confirmed by HPLC.

The dosing suspension was prepared by first concentrating the stock solution to near dryness under a nitrogen stream, then formulating in 0.5% aqueous Tragacanth using an ultrasonic bath. The suspension was then placed on a magnetic stirrer overnight at approximately 5°C, and then at room temperature during actual dosing.

After dosing, the radiochemical purity of the suspension was confirmed by HPLC analysis, while the amount of radioactivity was determined by LSC.

4. Dose administration

The concentration of the dosing suspension was calculated to achieve an administered amount of approximately 2 mg test material/kg bw, assuming an animal weight of 200 g.

The suspension was administered by oral gavage, using a feeding cannula. The amount and resulting radioactivity administered to each rat is shown below.

Administered per rat					
Fluo-picolide [mg]	Volume [mL]	Radioactivity [dpm]	Nominal dose [mg/kg bw]	Actual dose [mg/kg bw]	Mean rat weight [g]
0.36	0.9	75,889,369	2	2.04	176

5. Collection of samples

Urine, bile, and faeces samples were collected at various times separately for each animal. Skin, carcass and gastro-intestinal tract (GIT) were dissected from the animals at sacrifice and weighed. The collection intervals for the respective samples are shown below.

Time of collection after dosing (h)			
Urine	**Faeces**	**Bile**	**GIT**
0-4, 4-8, 8-24, 24-48	0-24, 24-48	0-4, 4-8, 8-24, 24-48	48

The animals were sacrificed using an overdose of pentobarbital-sodium.

6. Preparation and analysis of samples

The funnels for urine collection were rinsed into the corresponding urine fraction, then weighed prior to analysis. For bile, aliquots were taken for analysis. Faecal fractions were diluted with water, weighed, then homogenized and combusted prior to analysis. Skin, carcass and GIT were homogenized prior to taking aliquots for combustion and analysis.

Measurement of radioactivity in the liquid samples was carried out by LSC. All solid samples were combusted in an oxygen atmosphere using an oxidiser. The released $^{14}CO_2$ was absorbed in a special alkaline reagent. After mixing the reagent with liquid scintillator the radioactivity was determined by LSC:

For liquids, the volume of the entire sample was determined and aliquots (1-3 replicates) were subjected to LSC. The mean measurement efficiency was 86.59%. Solid samples were weighed and combusted in oxygen, the $^{14}CO_2$ being absorbed into Oxysolve scintillator liquid prior to LSC.

Counting times were generally from a few seconds to 20 min. Measurement was stopped after reaching a 2-sigma of 0.21% or after 20 min.

For all steps during sample preparation (extraction and clean-up), the values for individual fractions were normalized to 100% of recovered radioactivity.

For identification of parent and metabolites in the samples, the urine sample aliquots from all animals were combined into a representative pool, all sampling intervals being combined into a pool for 0-48 h. The bile samples were similarly pooled (0-48 h) for analysis. Owing to the low amount of radioactivity found in the faeces (mean <4% of recovered dose), parent and metabolites were not investigated for this matrix.

Reversed phase HPLC with an acetonitrile/buffered water gradient was used to analyse parent and metabolites in urine and bile. The metabolite fractions were isolated using an established HPLC method. Further purification was achieved by varying the column type or the solvent gradient. These same methods were used for profiling and quantification in the study with the pyridyl label.

7. Treatment and evaluation of data

The data were processed and evaluated using PhaLIMS 4.7 (Pharmacokinetic-LIMS). The data were checked for outliers using an outlier test and any such values were excluded from subsequent calculations. If more than one-third of values contributing to a mean were below the LOQ, the calculated mean was also set below LOQ and excluded from arithmetic means.

Radioactivity in percent of total dose administered was normalized to percentage of total radioactivity recovered. Concentrations represented active substance equivalents, so taking account of the parent compound and all radiolabelled metabolites, and were dose normalized.

An assumption of even distribution into the body organs was assumed, taking the animal weight at sacrifice as a measure of body volume. The percentage amounts in organs were obtained from the product of dose-normalized concentrations and the corresponding gamma-values (equivalent to percentage wet-weight contribution to the body weight).

If the mean values for fraction samples or subsets were calculated from values with <LLQ, half of the LLQ was used in place of the non-determined value.

For the HPLC analyses, all peaks corresponding to a signal approximately 2.5x background were integrated. The LOQ was set at the level of the LOD.

Mass spectroscopy (electrospray ionization) and/or NMR spectroscopy were deployed as appropriate.

Parent compound and metabolites were numbered with peak identities ID 1 to ID 30, based on retention time and spectroscopic behaviour.

II. Results and Discussion

1. Stock solution and dosing suspension

Purity of the test material in the stock solution was proven by HPLC, identity by LC-MS and LC-MS/MS. Radiochemical purity of the dosing suspension was checked by HPLC and found to be 99.94%.

The actual dose delivered was 2.04 mg/kg bw (nominal 2 mg/kg bw).

2. Balance of radioactivity

101.94% of the administered dose was recovered in the test.

Table 5.1.1- 98: Balance of radioactivity in excreta and tissue of rats following oral [phenyl-UL-^{14}C]-fluopicolide at nominal 2 mg/kg bw, Mean and CV (coefficient of variation)

	Percent dose administered	CV		Percent dose recovered	CV
Bile	91.84	4.7		90.07	3.3
Faeces	3.74	58.6		3.69	59.2
Urine	4.01	30.8		3.94	31.6
Total excreted	**99.59**	2.2		**97.69**	0.3
Body excluding GIT	2.204	13.2		2.159	11.5
GIT	0.152	73.4		0.148	69.7
Total in body	**2.357**	16.2		**2.307**	14.1
Balance	**101.94**	2.5	**Normalization factor**	0.981	2.4
			Absorption (%)	**96.17**	2.2

3. Absorption

In the time period from 0 (dosing) to 48 h post-dose the total absorbed was 96.2% of recovered dose (total in urine, bile and body without the GIT), while 90.1% was detected in the bile.

4. Excretion

The major part of the radioactivity was detected in the bile. At 24 h after administration, more than 96% of the recovered dose had been excreted in bile, faeces, and urine (approximately 4% in urine, 3.7% in the faeces).

Table 5.1.1- 99: Excretion of radioactivity (% of dose administered and recovered), Mean and coefficient of variation (CV) after oral 2 mg/kg bw

Time (h post dose)	% of dose administered	CV	% of dose recovered	CV
Bile				
4	46.91	28.7	45.81	26.7
8	76.23	7.3	74.73	5.8
24	90.82	4.8	89.06	3.1
48	91.84	4.7	90.07	3.3
Faeces				
24	3.59	62.5	3.53	63.1
48	3.74	58.6	3.69	59.2
Urine				
4	0.70	89.8	0.69	89.9
8	2.44	46.2	2.39	47.1
24	3.70	32.8	3.64	33.7
48	4.01	30.8	3.94	31.6
Sum excreted:	**99.59**	2.2	**97.69**	0.3
Normalization factor			0.981	2.4

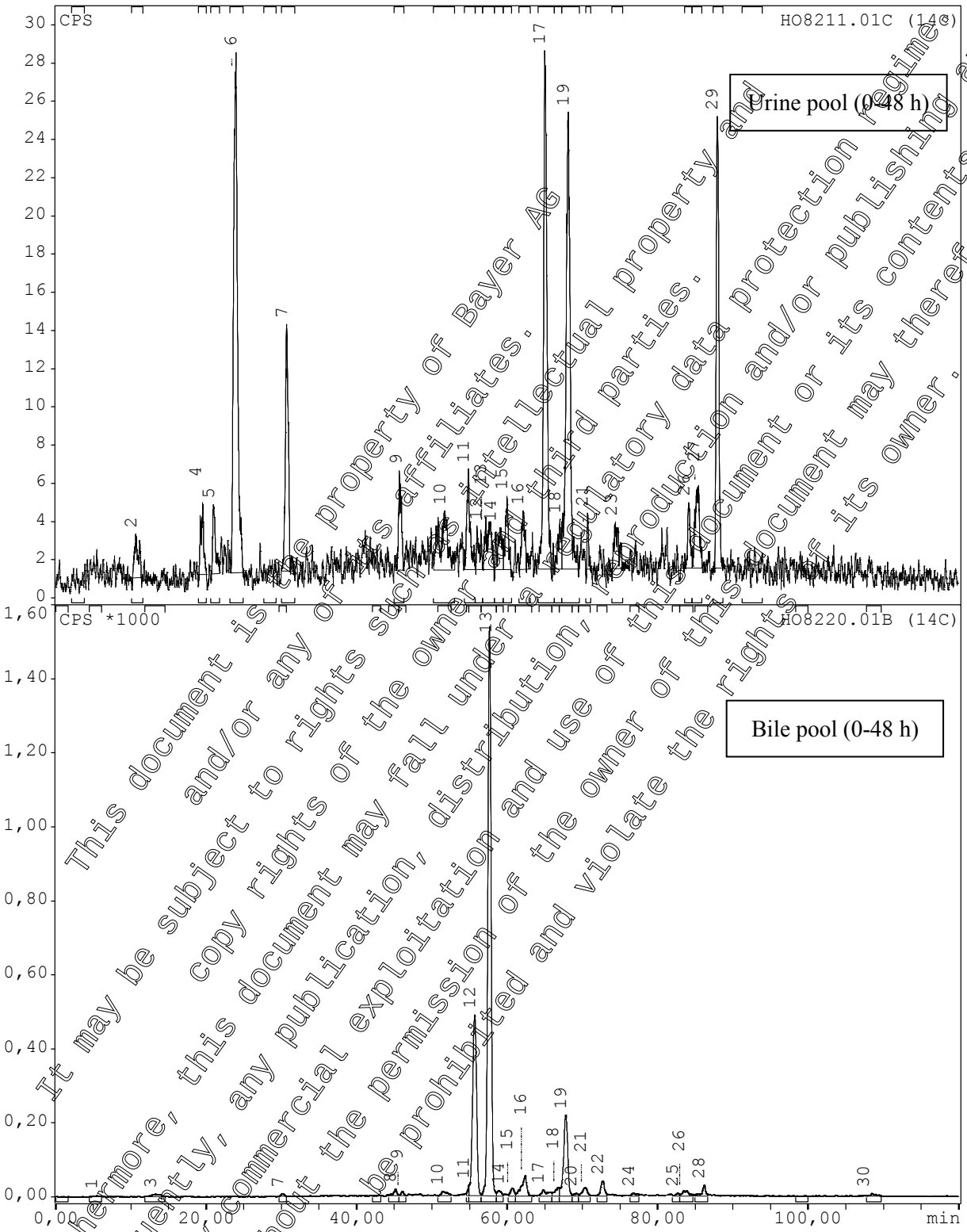
5. Residues in the carcass and GIT

At sacrifice, only low levels of radioactivity were found in the bodies excluding GIT (2.20% of the recovered dose). Only 0.152% of radioactivity was detected in the GIT.

6. Metabolism

Metabolite profiles showed extensive metabolic transformation. The profile for bile and urine is shown below. Faecal samples were not analysed, owing to the low amount of radioactivity detected in that matrix.

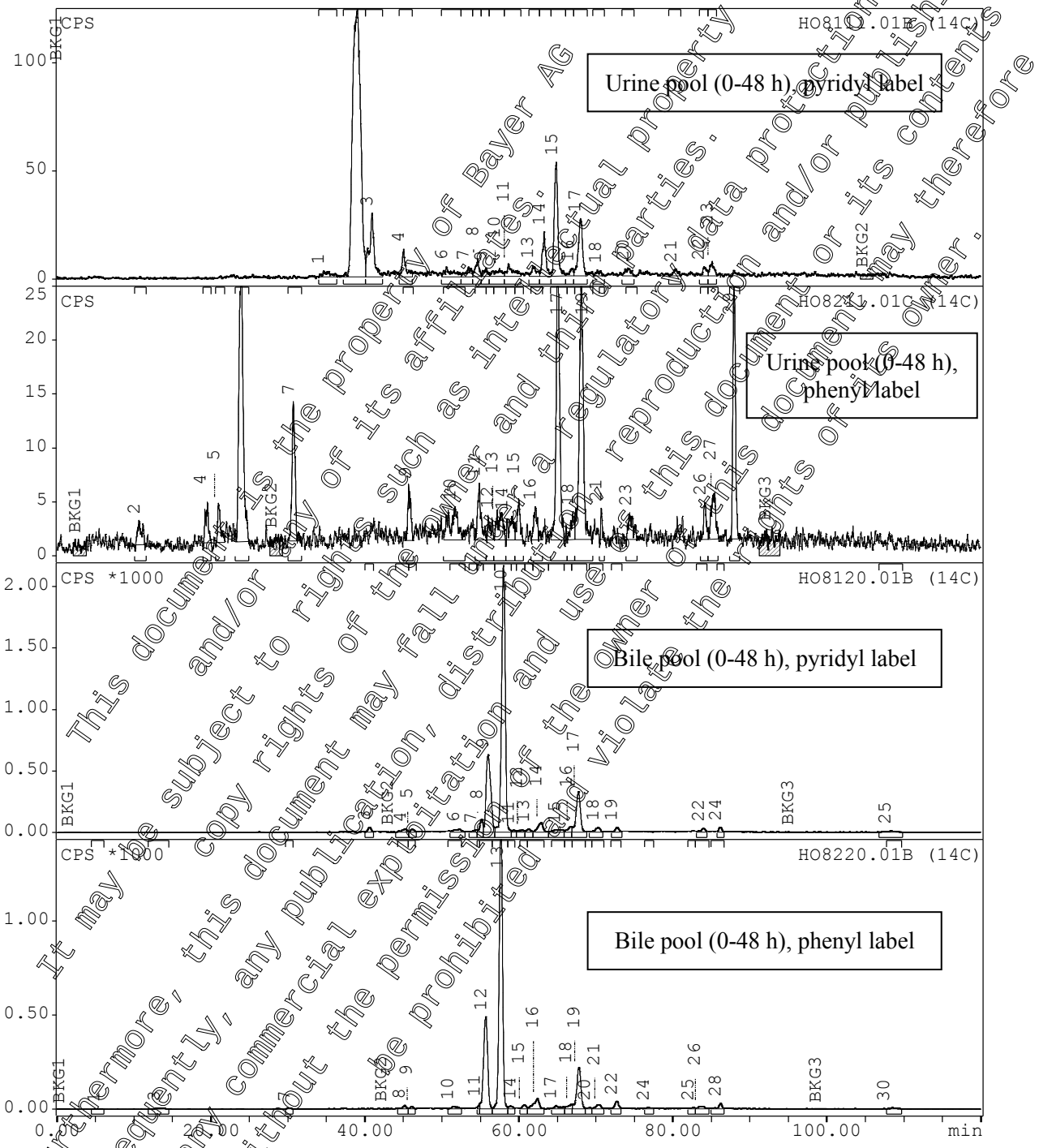
Figure 5.1.1-23: Comparison of the ^{14}C-HPLC chromatograms of urine and bile from male rats



Note: Metabolites with the same identified chemical structure in urine and bile are shown joined with drawn vertical lines

Label-specific differences were observed between urine samples of the current study and the corresponding rat study with [pyridyl-2,6-^{14}C]-fluopicolide. The metabolite profile of bile samples was comparable in both studies. A comparison is shown in the figure below.

Figure 5.1.1-24:Comparison of ^{14}C-chromatograms of urine and bile from male rats



The peak IDs (based on retention times) and identification of the metabolites from the phenyl label is shown in the following tables:

Table 5.1.1- 100: Overview of identified and characterized metabolites in urine and bile of male rats (phenyl label) after oral 2 mg/kg bw

Peak ID	Appearance in		Report name
	urine	bile	
1	---	X	unknown 1
2	X	---	unknown 2
3	---	X	unknown 3
4	X	---	unknown 4
5	X	---	unknown 5
6	X	---	unknown 6
7	X	X	BAM (2,6-dichlorobenzamide)
8	---	X	unknown 7
9	X	X	unknown 8
10	X	X	unknown 9
11	X	X	unknown 10
12	X	X	HO812103
13	X	X	HO812104
14	X	X	unknown 11
15	X	X	unknown 12
16	X	X	HO812106
17	X	X	unknown 13
18	X	X	unknown 14
19	X	X	HO812105
20	---	X	unknown 15
21	X	X	unknown 16
22	---	X	unknown 17
23	X	X	unknown 18
24	---	X	unknown 19
25	---	X	unknown 20
26	X	X	AE C643890 (3-OH 206)
27	X	---	unknown 21
28	---	X	unknown 22
29	X	---	Fluopicolide
30	---	X	unknown 23

X = detected; --- = not detected

Table 5.1.1- 101: Quantitation of metabolites in urine of male rats (phenyl label) after oral 2 mg/kg bw

Peak ID	Report name	Rt (min)	Area (%)	% of dose
2	unknown 2	10.7	1.48	0.06
4	unknown 4	19.5	1.90	0.08
5	unknown 5	21.0	1.74	0.07
6	unknown 6	23.9	18.23	0.73
7	BAM (2,6-dichlorobenzamide)	30.7	6.39	0.26
9	unknown 8	45.9	2.52	0.10
10	unknown 9	51.7	4.30	0.17
11	unknown 10	54.9	2.97	0.12
12	HO812103	56.5	1.17	0.05
13	HO812104	57.1	2.47	0.10
14	unknown 11	58.5	1.77	0.07
15	unknown 12	60.0	1.89	0.08
16	HO812106	62.1	1.89	0.08
17	unknown 13	65.0	15.80	0.63
18	unknown 14	67.0	1.35	0.05
19	HO812105	68.1	16.41	0.66
21	unknown 16	70.7	0.65	0.03
23	unknown 18	74.5	1.91	0.08
26	AE C643800 (3-OH 206)	84.2	1.56	0.06
27	unknown 21	85.5	3.25	0.13
29	Fluopicolide	88.0	10.37	0.42
Total				**4.01**
Sum of identified				**1.61**
Sum of characterized				**2.40**

Table 5.1.1- 102: Quantitation of metabolites in bile of male rats (phenyl label) after oral 2 mg/kg bw

Peak ID	Report name	Rt (min)	Area (%)	% of dose
1	unknown 1	5.4	0.10	0.09
3	unknown 3	13.3	0.54	0.50
7	BAM (2,6-dichlorobenzamide)	30.2	0.19	0.17
8	unknown 7	45.1	0.64	0.59
9	unknown 8	46.1	0.35	0.32
10	unknown 9	51.4	0.77	0.71
11	unknown 10	54.9	0.48	0.44
12	HO812103	55.7	20.67	18.98
13	HO812104	57.6	53.98	49.57
14	unknown 11	59.5	0.60	0.55
15	unknown 12	60.6	0.81	0.74
16	HO812106	62.4	3.01	2.76
17	unknown 13	64.8	1.03	0.95
18	unknown 14	66.7	1.08	0.99
19	HO812105	67.8	9.53	8.75
20	unknown 15	69.2	0.28	0.26
21	unknown 16	70.4	1.17	1.07
22	unknown 17	72.7	1.63	1.50
24	unknown 19	76.8	0.40	0.37
25	unknown 20	82.7	0.29	0.27
26	AE C643890 (3-OH 2061)	83.5	0.96	0.88
28	unknown 22	86.2	1.14	1.05
30	unknown 23	108.5	0.35	0.32
Total				**91.84**
Sum of identified				**81.13**
Sum of characterized				**10.71**

Parent compound amounted to 0.42% of the administered dose in the urine of male bile-duct cannulated rats.

Metabolites HO812104 (49.57% of dose) and HO812103 (18.98% of dose) - cysteine and cysteine-glycine conjugates of 3-OH 206 - were detected as major metabolites in bile samples.

Metabolite HO812105 (a glucuronic acid conjugate of 3-OH 206) was identified in bile (8.75% of dose).

Several minor metabolites amounted to <3.0% of dose and were identified as HO812106, 3-OH 206 and BAM (2,6-dichlorobenzamide).

All metabolites observed in bile were also detected to low amounts in the urine.

More metabolites were indicated by broad non-resolved zones in the chromatograms. Unidentified metabolites were characterised by their extraction and chromatographic behaviour.

All results correspond well with the findings in the ADME study following administration of [pyridyl-2,6-^{14}C]-fluopicolide. The metabolite pattern in the urine samples from the current study and the companion study with the pyridyl label was similar, except for the individual label-specific metabolites.

Label-specific metabolites

Elimination of the pyridyl moiety led to phenyl label specific metabolites which could not be detected in the pyridyl label study. The metabolite BAM occurred as a label-specific metabolite in urine and bile samples in the current study and amounted to 0.43% (in total) of the dose administered.

7. Metabolic pathway

In general, the main metabolic routes observed for rats treated with [phenyl-UL-^{14}C]-fluopicolide and [piridyl-2,6-^{14}C]-fluopicolide were identical. Low levels of label-specific metabolites were identified.

A large number of metabolites was identified and characterised in the urine and bile. A low amount of the parent compound fluopicolide was detected in urine samples, this with only a small proportion of the radioactivity in faeces samples, indicates extensive metabolism of fluopicolide in the rat.

The main metabolic reaction of [phenyl-UL-^{14}C]-fluopicolide was hydroxylation of the phenyl ring leading to AE C643890 (3-OH 206). Overall, hydroxylation in position 3 of the phenyl ring was observed in 79.04% of the dose administered.

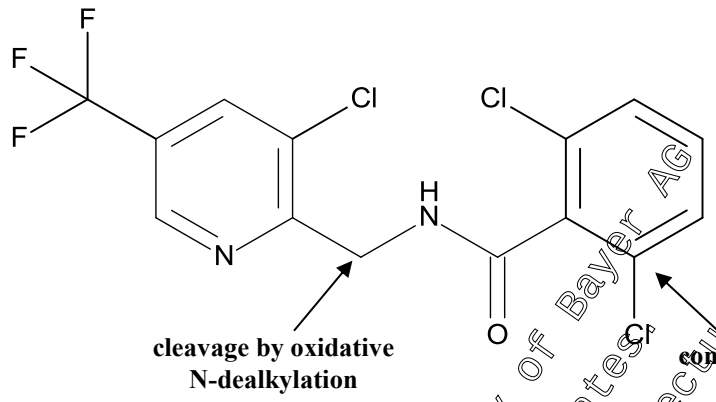
Metabolite 3-OH 206 was identified and resulted also in conjugates with cysteine, cysteine-glycine cysteine (via glutathione conjugation and subsequent loss of glycine and glutamic acid) and glucuronic acid. Conjugation was the most important metabolic reaction in the elimination process for [phenyl-UL-^{14}C]-fluopicolide.

Cleavage of the amide bond by oxidative N-dealkylation was observed as a minor reaction.

The principal metabolic reactions of [phenyl-UL-^{14}C]-fluopicolide in the rat are listed below:

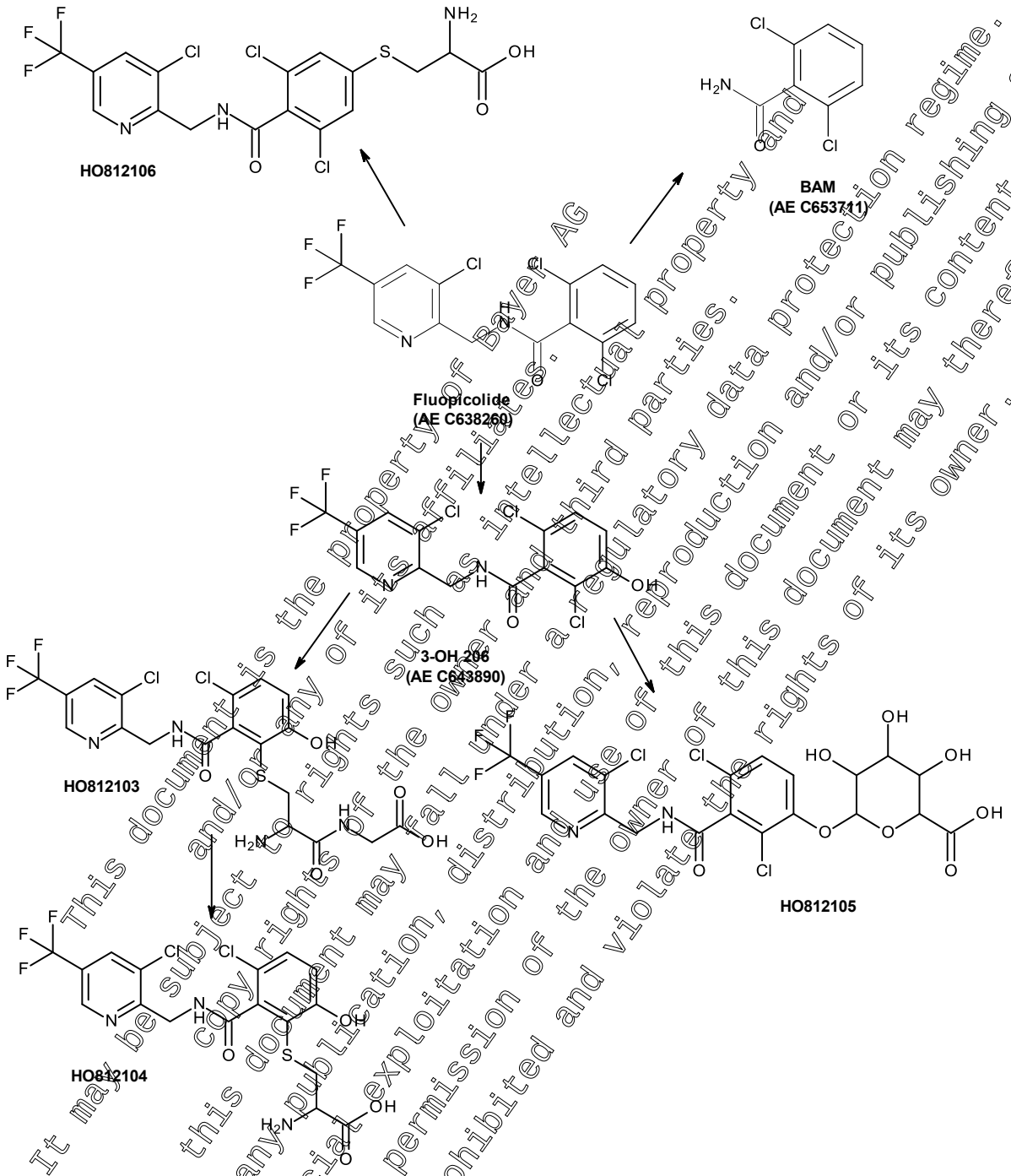
- hydroxylation in position 3 of the phenyl ring leading to 3-OH 206
- subsequent conjugation with glucuronic acid in position 3 of the phenyl ring to 3-OH 206
- conjugation of 3-OH 206 in position 2 of the phenyl ring with cysteine or with cysteine-glycine via glutathione conjugation and subsequent loss of glutamic acid
- conjugation with cysteine (via glutathione conjugation) in position 4 of the phenyl ring of the parent molecule
- cleavage of the parent molecule at the amide bond by oxidative N-dealkylation

The figure below schematically shows the positions in the molecule that are mainly involved in the metabolic reactions:



The proposed biotransformation pathway of [phenyl-UL-^{14}C]-fluopicolide is presented in the figure below.

Figure 5.1.1-25: Proposed metabolic pathway of [phenyl-UL-^{14}C]-fluopicolide in the rat



III. Conclusions

The excretion and metabolic behaviour of [phenyl-UL-^{14}C]-fluopicolide in bile-duct cannulated male rats may be characterized as follows:

- Fluopicolide shows a high absorption rate in male rats following administration of 2 mg/kg bw. At 48 h post-dose the recovered dose amounted to about 96%.
- Excretion was predominantly via the bile, with about 92% of the administered radioactivity found in the bile and about 4% in the urine.
- At sacrifice about 2.2% of the administered radioactivity was found in the bodies excluding GIT, a low amount of radioactivity remaining in the GIT (<0.2%).
- Absorbed radioactivity was quickly and efficiently eliminated from the bodies of the rats. Elimination was nearly complete at sacrifice 48 hours after administration, by which time point >97% of the recovered dose was eliminated.
- Only small amounts of parent compound were excreted with the urine.
- Six metabolites were identified in bile and urine of male rats. The metabolites HO812104 (49.67% of the administered dose) and HO812103 (19.03%) were found as major metabolites in male rats (cysteine and cysteine-glycine conjugates of 3-OH 206).
- Minor metabolites were HO812105 (9.41%) and HO812106 (2.84%). The metabolites 3-OH 206 and BAM (2,6-dichlorobenzamide) were also identified, amounting to less than 2% of dose.
- The most important metabolic route of [phenyl-UL-^{14}C]-fluopicolide was hydroxylation of the phenyl ring leading to 3-OH 206, followed by cysteine and cysteine-glycine conjugation (via glutathione) in position 3 of the phenyl ring.
- Conjugation was the most important metabolic reaction in the elimination process of [phenyl-UL-^{14}C]-fluopicolide. Conjugation of 3-OH 206 with cysteine, cysteine-glycine (via glutathione) and glucuronic acid, as well as conjugation of the parent compound with cysteine was observed.
- Cleavage of the amide bond by oxidative N-dealkylation was observed as a minor reaction.

The results are in good agreement with the results of the companion study in bile duct cannulated male rats with [pyridyl-2,6-^{14}C]-fluopicolide (see preceding study summary).

Based on these results the metabolic behaviour of [phenyl-UL-^{14}C]-fluopicolide in male rats is well understood.

Assessment and conclusion by applicant:
An acceptable study yielding valid conclusions

CA 5.1.2 Absorption, distribution, metabolism and excretion by other routes

No studies relating to absorption, distribution, metabolism, and excretion by other routes are available.

CA 5.2 Acute toxicity

The acute oral LD_{50} of fluopicolide in rats was > 5,000 mg/kg bw. Clinical signs of toxicity included piloerection, which was observed in all rats within 1 – 2½ hours of dosing. At later time-points on day 1 of dosing, piloerection, accompanied only by hunched posture and abnormal gait was observed. Recovery was complete by Day 3.

The acute dermal LD_{50} of fluopicolide in rats was > 5,000 mg/kg bw.

The 4-hour acute inhalation LC_{50} of fluopicolide in rats was > 5.16 mg/L (the mean achieved concentration).

Fluopicolide was not irritating to rabbit skin. No dermal irritation was observed during the study. The mean irritation scores over 24 – 72 hours were 0 for both erythema and oedema.

Fluopicolide was transiently slightly irritating to the rabbit eye. The ocular reactions were very slight and had resolved in all instances within two days following instillation.

Fluopicolide was not a skin sensitizer in a guinea pig Magnusson and Kligman test.

Therefore, no classification is required for the acute toxicity of fluopicolide when comparing the study results / derived LD_{50} and LC_{50} values with CLP criteria according to the Regulation (EC) No 1272/2008[10].

According to the CLP criteria a classification for STOT-SE needs to be considered if the substance causes non-lethal target organ toxicity after a single exposure (i.e. significant health effects that can impair function, both reversible and irreversible, immediate and/or delayed and not covered by acute toxicity, skin corrosion / irritation, eye damage / irritation, respiratory or skin sensitisation, genotoxicity, carcinogenicity and reproductive toxicity should be taken into consideration).

Based on the results after acute exposure to fluopicolide in toxicological studies with single and repeated dosing, no significant toxic effects on specific target organs were observed at non-lethal dose levels. Thus, classification of fluopicolide for STOT-SE Category 1 or 2 is not warranted.

There is also no indication of transient effects like respiratory tract irritation (RTI) and narcotic effects (NE) after single exposure to fluopicolide. Classification of fluopicolide for STOT-SE Category 3 is therefore also not warranted.

No classification / labelling is warranted regarding Specific Target Organ Toxicity – Single Exposure (STOT-SE) for fluopicolide when comparing the study results with CLP criteria according to the Regulation (EC) No 1272/2008[10].

Fluopicolide was discussed at the 53rd meeting of the Committee for Risk Assessment (RAC-53). It was agreed that fluopicolide should not be classified for acute toxicity *via* the oral, dermal or inhalation routes, skin, or eye irritation or skin sensitisation. It was also agreed that classification for STOT SE was not warranted.

[10] Regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006. Version 5.0 July 2015

Table 5.2- 1: Summary of acute toxicity studies

Method Guideline GLP compliance	Test substance Purity Solvent/Vehicle	Result	Classification ((EC) No 1272/2008)	Reference
Rat acute oral toxicity 5 Sprague Dawley rats/sex OECD 423 (1996) GLP	Fluopicolide (purity 97.7%) in 1% w/v aqueous methylcellulose	LD_{50} > 5000 mg/kg bw in M/F	none	[redacted] 2000; M-197224-01-1
Rat acute dermal toxicity 5 Sprague Dawley rats/sex OECD 402 (1987) GLP	Fluopicolide (purity 97.7%) in 1% w/v aqueous methylcellulose	LD_{50} > 5000 mg/kg bw in M/F	none	[redacted] 2000; M-197225-01-1
Rat acute inhalation toxicity (4 hours) 5 Sprague Dawley rats/sex OECD 403 (1981) GLP	Fluopicolide (purity 98.3%) as dust	LC_{50} inhalation > 5.16 mg/L	none	[redacted] 2000; M-197229-01-1
Rabbit skin irritancy 3 female New Zealand White rabbits OECD 404 (1992) GLP	Fluopicolide (purity 97.7%) moistened with distilled water	The mean irritation score (24-72 hours) was 0.0	none	[redacted] 2000; M-197226-01-1
Rabbit eye irritancy 4 female New Zealand White rabbits OECD 405 (1987) GLP	Fluopicolide (purity 97.7%)	Mean scores for 24, 48- and 72-hours post instillation were 0.0 for all parameters, with the exception of conjunctival redness, which had a score of 0.33. Reactions had resolved in all instances two days after instillation.	none	[redacted] 2000; M-197227-01-1
Guinea pig skin sensitization study Dunkin/Hartley guinea pigs 20 animals (treatment) + 10 animals (control) OECD 406 (1992) GLP	Fluopicolide (purity 97.7%) in sterile water	No specific skin responses compared to control Not a skin sensitiser	none	[redacted] 2000; M-197228-01-1
Phototoxicity	-	Considered to be not required due to its ultraviolet/visible molar extinction/absorption properties	-	-

M = male F = female

CA 5.2.1 Oral

Data Point:	KCA 5.2.1/01
Report Author:	[redacted]
Report Year:	2000
Report Title:	Rat acute oral toxixity Code: AE C638206 00 1C99 0005
Report No:	C008135
Document No:	M-197224-01-1
Guideline(s) followed in study:	Directive 96/54/EEC, B,1 (1996); OECD: 423 (1996)
Deviations from current test guideline:	Five animals/sex rather than three/sex were used in the limit test
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

In an acute toxicity study conducted according to the acute toxin class method, five male and five female fasted Sprague-Dawley rats were each administered a single oral gavage dose of 5,000 mg/kg bw of fluopicolide.

No mortality was observed. Clinical signs of reaction to treatment were confined to piloerection and hunched posture, seen in all female rats and in three male rats, with abnormal gait notable in three females. Recovery of rats, as judged by external appearance and behaviour, was complete by Day 3. All animals were considered to have achieved satisfactory body weight gains throughout the study.

In conclusion, the acute median lethal oral dose (LD_{50}) of fluopicolide in rats was greater than 5,000 mg/kg bw.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 (fluopicolide)
Purity:	97.7%
Batch no.:	PP/241024/2 and PP/241067/1

2. Vehicle and/or positive control

Vehicle:	1% w/v aqueous methylcellulose

3. Test animals

Species:	Rat
Strain:	Male and female CD rats of Sprague-Dawley origin (Hsd: Sprague-Dawley(CD))
Age:	8-11 weeks
Weight at start:	195 g (186 – 201 g) in males and 206 g (196 – 220 g) in females
Source:	[redacted]
Acclimation period:	Yes
Diet:	Special Diet Services RM1(E) SQC expanded pellet
Water:	Water *ad libitum*
Housing:	In groups of up to five rats of the same sex in metal cages (RS Biotech Sub-Dividable Rodent Cages - polished stainless steel (20 cm high x 39 cm wide x 39 cm long)).
Temperature:	21 – 22 °C
Humidity:	30 – 50%
Air changes:	Not given
Photoperiod:	12 hours

B. Study design

1. In-life dates: April 3 to April 19, 2000

2. Animal assignment and treatment

A group of ten rats (five males and five females) was treated at 5000 mg/kg bw, a dosage selected after review of preliminary study results with administration of a dose of 4000 mg/kg bw. The appropriate dose volume of the test substance was administered to each rat by oral gavage using a plastic syringe and catheter of the appropriate gauge. The day of dosing was designated Day 1.

C. Methods

1. Observations

Mortality:

Cages of rats were checked at least twice daily for mortalities.

Clinical signs:

Animals were observed soon after dosing and at frequent intervals for the remainder of Day 1. On subsequent days animals were observed on two occasions during the day (once in the morning and again at the end of the experimental day, with the exception of the day of study termination – morning only). The nature and severity of clinical signs and the time these were noted were recorded at each observation. Animals in the preliminary and main study were observed for 7 or 14 days respectively following dosing.

The body weights of each rat in the preliminary study were recorded on Days 1 (prior to dosing) and 8 and in the main study on Days 1 (prior to dosing), 8 and 15.

2. Necropsy

All animals were killed by carbon dioxide asphyxiation at study termination.

Animals were subjected to a macroscopic examination, which consisted of opening the cranial, thoracic and abdominal cavities. The macroscopic appearance of all examined organs was recorded.

II. Results and Discussion

A. Results

1. Dose-response table (LD_{50})

The results of the study for acute oral toxicity in the fasted rat, including the LD_{50}, are summarized in Table 5.2.1- 103.

The LD_{50} was therefore **> 5,000 mg/kg bw**

Table 5.2.1- 103: Dose response

Dose (mg/kg bw)	Toxicological result*	Occurrence of signs	Time of death	Mortality (%)
Males				
Prelim. 4,000	0/1/1	1/1	-	0
5,000	0/5/5	5/5	-	0
Females				
Prelim. 4,000	0/1/1	1/1	-	0
5,000	0/5/5	5/5	-	0

* Number of animals which died and/or were sacrificed moribundly / number of animals with signs / total number of animals.

2. Clinical signs

Clinical signs of reaction to treatment were confined to piloerection and hunched posture, seen in all female rats and in 3/5 males, with abnormal gait notable in 3/5 females. Recovery of rats was complete by Day 3.

3. Body weights

There were no treatment-related effects on body weight or body weight gain.

4. Necropsy findings

The necropsies performed at the end of the study did not reveal any unusual findings.

III. Conclusion

The acute oral LD_{50} of fluopicolide in rats was greater than 5,000 mg/kg bw.

Assessment and conclusion by applicant:

Study was conducted in accordance with OECD 423 and is acceptable to determine the acute oral toxicity of fluopicolide. An LD50 of >5000 mg/kg bw was determined from this study.

CA 5.2.2 Dermal

Data Point:	KCA 5.2.2/01
Report Author:	[redacted]
Report Year:	2000
Report Title:	Rat acute dermal toxicity Code: AE C638206 00 1C99 0005
Report No:	C008136
Document No:	M-197225-01-1
Guideline(s) followed in study:	JMAFF 59 NohSan No. 4200 (1985); Directive 92/69/EEC Part B, B.3. (1992); OECD 402 (1987); US-EPA 712-C-98-192, OPPTS 870.1200 (1998)
Deviations from current test guideline:	Five animals/sex rather than three/sex were used in the limit test and all animals were treated concurrently rather than sequentially
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

A group of ten rats (five males and five females) received a single topical application of the test substance, administered as supplied at a dose level of 5,000 mg/kg bw of fluopicolide. The application site was occluded for 24 hours. All animals were observed daily for 14 days and body weights were recorded at weekly intervals post dosing. The animals were killed as scheduled at study termination (Day 15) and subjected to a macroscopic examination.

No mortality was observed. There were no clinical signs of reaction to treatment observed in any animal throughout the study.

There was no evidence of a dermal response to treatment observed in any animal throughout the study.

A slight reduced body weight was evident in 2/5 females on day 8. All other animals were considered to have achieved satisfactory body weight gains throughout the study.

No macroscopic abnormalities were observed for animals killed at study termination on Day 15.

The acute median lethal dermal dose (LD_{50}) to rats of fluopicolide was greater than 5,000 mg/kg bw.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 (fluopicolide)
Purity:	97.7%
Batch no.:	PP/241024/2 and PP/241067/1

2. Vehicle and/or positive control

Vehicle:	1% w/v aqueous methylcellulose

3. Test animals

Species:	Rat
Strain:	Male and female CD rats of Sprague-Dawley origin (Hsd: Sprague Dawley(TM) CD))
Age:	8-11 weeks
Weight at start:	Males: 247 g (231 – 263 g), females: 228 g (220 – 233 g)
Source:	[redacted]
Acclimation period:	Yes
Diet:	Special Diet Services RM1(E) SQC expanded pellet
Water:	Water *ad libitum*
Housing:	In groups of up to five rats of the same sex in metal cages (RS Biotech Sub-Dividable Rodent Cages - polished stainless steel (20 cm high x 39 cm wide x 39 cm long)).
Temperature:	19 - 21 °C
Humidity:	24 – 50%
Air changes:	Not given
Photoperiod:	12 hours

B. Study design

1. In-life dates: February 1 to February 17, 2000

2. Animal assignment and treatment

A preliminary study comprising two rats (one male and one female) dosed at 3,200 mg/kg bw was conducted to help define the toxic potential of the test substance.

In the main study a group of ten rats (five males and five females) received a single dermal administration of the test substance at a dose level of 5,000 mg/kg bw.

In the main study, the test substance was applied to the closely clipped dorsum of each animal on day 1 at a dosage of 5,000mg/kg bw and covered by an occlusive dressing for 24 hours. In order to achieve the desired dose concentration, fluopicolide was formulated at a maximum practical concentration of 100% w/v in 1% w/v aqueous methylcellulose and administered at a dose volume of 5 mL/kg bw. The test substance was prepared on the day of dosing. The test substance was applied by spreading evenly over the prepared skin. The treatment area (approx. 50 mm x 50 mm) was covered with porous gauze held in place with a non-irritating dressing, and further covered by a waterproof dressing encircled firmly around the trunk of the animal. At the end of the 24-hour exposure period the dressings were carefully removed, and the treated area of skin was washed with warm water (30-40 °C) to remove any residual test substance. The treated area was then blotted dry with absorbent paper.

C. Methods

1. Observations

Mortality:

Cages of rats were checked at least twice daily for mortalities.

Clinical signs:

Animals were observed soon after dosing and at frequent intervals for the remainder of Day 1. On subsequent days animals were observed on two occasions during the day (once in the morning and again at the end of the experimental day, with the exception of the day of study termination on which morning examinations only were conducted). The nature and severity of clinical signs and the time these were noted were recorded at each observation. Animals in the preliminary and main study were observed for 7 or 14 days respectively following dosing.

Local dermal irritation at the treatment site was assessed daily.

The body weights of each rat in the preliminary study were recorded on Days 1 (prior to dosing) and 8 and in the main study on Days 1 (prior to dosing), 8 and 15 (or at death).

2. Necropsy

All animals were killed by carbon dioxide asphyxiation at study termination.

Animals were subjected to a macroscopic examination, which consisted of opening the cranial, thoracic and abdominal cavities. The macroscopic appearance of all examined organs was recorded.

II. Results and Discussion

A. Results

1. Dose-response table (LD_{50})

The results of the study for acute oral toxicity in the fasted rat, including the LD_{50}, are summarized in Table 5.2.2- 1.

The LD_{50} dermal was therefore **> 5,000 mg/kg bw**

Table 5.2.2- 1: Dose response

Dose (mg/kg bw)	Toxicological result*	Occurrence of signs	Time of death	Mortality (%)
Males				
Prelim. 3,200	0/0/1	0/1	-	0
5,000	0/0/5	0/5	-	0
Females				
Prelim. 4,000	0/0/1	0/1	-	0
5,000	0/0/5	0/5	-	0

* Number of animals which died, and/or were sacrificed moribundly / number of animals with signs / total number of animals.

2. Clinical signs

There were no deaths following a single dermal application of fluopicolide to a group of ten rats (five males and five females) at a dose level of 5,000 mg/kg bw.

There were no clinical signs of reaction to treatment observed in any animal throughout the study.

There was no evidence of a dermal response to treatment observed in any animal throughout the study.

3. Body weights

A slight body weight loss was evident in two females on Day 8. All other animals were considered to have achieved satisfactory body weight gains throughout the study.

4. Necropsy findings

No macroscopic abnormalities were observed for animals killed at study termination on Day 15.

III. Conclusion

The acute dermal LD_{50} to rats of fluopicolide was greater than 5,000 mg/kg bw.

Assessment and conclusion by applicant:

Study was conducted according to OECD TG 402 and is acceptable to determine the acute dermal toxicity of fluopicolide. An LD50 of >5000 mg/kg bw was determined rom this study.

CA 5.2.3 Inhalation

Data Point:	KCA 5.2.3/01
Report Author:	[redacted]
Report Year:	2000
Report Title:	Rat acute inhalation toxicity Code: AE C638206 00 1C99 0005
Report No:	C008140
Document No:	M-197229-01-1
Guideline(s) followed in study:	Directive 67/548/EEC, B.2; JMAFF 59 NohSan No. 4200 (1985); OECD 403 (1981); US-EPA OPPTS 870.1300 (1998)
Deviations from current test guideline:	Method: Deviations from current guideline SANCO/3029/99 rev. 4: For the accuracy and precision data, there are only 4 recoveries in total at 1 fortification level (LOQ samples). However, the mean accuracy is between 70-110% and RSD < 20%. Considering that this analytical method is validated in support of a toxicological cold study, the method validation is considered fit for purpose.; Study: None
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

The acute inhalation toxicity of fluopicolide was investigated by exposing a group of five male and five female Sprague-Dawley (CD) rats to a dust atmosphere containing the limit concentration of the test substance (5 mg/L). The test group was subjected to a single 4-hour continuous, nose only exposure. Signs of reaction to treatment were recorded during a subsequent 14-day observation period. The animals were sacrificed at the end of the observation period and were subject to a detailed necropsy.

No mortality was recorded. Common observations noted both during and post exposure included wet fur, hunched posture, piloerection and increased respiratory rate. Isolated occurrences of noisy respiration and red/brown staining around the snout or eyes were also seen. Animals recovered quickly and appeared normal on the first day following exposure.

The animals gained the expected amount of weight during the study.

No macroscopic abnormalities were noted for 9/10 animals; however, one male presented with dark foci on the lungs.

In conclusion, the 4-hour acute inhalation LC_{50} of fluopicolide in rats was > 5.16 mg/L (the mean achieved concentration).

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 (fluopicolide)
Purity:	98.3%
Batch no.:	PP/241024/2 and PP/241067/1

2. Vehicle and/or positive control

Vehicle:	None

3. Test animals

Species:	Rat
Strain:	Sprague-Dawley Crl:CD®BR
Age:	8-10 weeks
Weight at start:	Males: 295 to 317 g, females: 230 to 243 g
Source:	[redacted]
Acclimation period:	Yes
Diet:	Rat and Mouse Expanded Diet No. 1, Special Diets Services Limited, Witham, Essex, UK)
Water:	Water *ad libitum*
Housing:	In groups of five by sex in solid-floor polypropylene cages with stainless steel lids, furnished with softwood flakes (Dates and Ltd, Cheshire, UK).
Temperature:	21 ± 2 °C
Humidity:	55 ± 15%
Air changes:	At least 15/hour
Photoperiod:	12 hours

B. Study design

1. In-life dates: February 21 to March 23, 2000

2. Animal assignment and treatment

The acute inhalation toxicity of fluopicolide was investigated by exposing a group of five male and five female Sprague-Dawley (CD) rats to a dust atmosphere containing the limit concentration (5 mg/L) of the test substance. The test group was subjected to a single 4-hour continuous, nose only exposure. Signs of reaction to treatment were recorded during a subsequent 14-day observation period. The animals were sacrificed at the end of the observation period and were subject to a detailed necropsy.

Table 5.2.3- 1: Actual concentration and atmosphere characteristics in chamber

Mean concentration ± SD (mg/L)	Nominal concentration (mg/L)	MMAD ± GSD (µm)	Resp. fraction (% < 4 µm)
5.16 ± 0.38	9.09	3.37 ± 2.09	59.1

C. Methods

1. Observations

All animals were observed for clinical signs at hourly intervals during exposure, immediately on removal from the restraining tubes at the end of exposure, one hour after termination of exposure and subsequently once daily, for fourteen days. Any evidence of overt toxicity was recorded at each observation.

Individual body weights were recorded prior to treatment on the day of exposure and on Days 7 and 14.

2. Necropsy

At the end of the 14-day observation period, the animals were killed by intravenous overdose of sodium pentobarbitone. All animals were subjected to a full external and internal examination, and any macroscopic abnormalities were recorded. The respiratory tract was subjected to a detailed macroscopic examination for signs of irritancy and/or local toxicity.

II. Results and Discussion

A. Results

1. Dose-response table (LC_{50})

The results of the study for acute inhalation toxicity in the fasted rat, including the LC50, are summarized in Table 5.2.3- 2.

Therefore, the LC_{50} inhalation is **> 5.16 mg/L**.

Table 5.2.3- 2: Dose response

Dose (mg/L)	Toxicological result*	Occurrence of signs	Time of death	Mortality (%)
Males				
5.16	0/5/5	5/5	-	0
Females				
5.16	0/5/5	5/5	-	0

* Number of animals which died and/or were sacrificed moribundly / number of animals with signs / total number of animals.

2. Clinical signs, mortality

There was no mortality during the exposure and observation period. Common observations noted both during and post exposure included wet fur, hunched posture, piloerection and increased respiratory rate. Isolated occurrences of noisy respiration and red/brown staining around the snout or eyes were also seen. Animals recovered quickly and appeared normal on the first day following exposure.

3. Body weights

The animals gained the expected amount of weight during the study.

4. Necropsy findings

No macroscopic abnormalities were noted for 9/10 animals; however, one male presented with dark foci on the lungs.

III. Conclusion

The 4-hour acute inhalation LC_{50} of fluopicolide in rats was > 5.16 mg/L (the mean achieved concentration).

Assessment and conclusion by applicant:
Study was conducted according to OECD TG 403 and is acceptable to determine the acute inhalation toxicity of fluopicolide. An LC50 of > 5.16 mg/L was determined from this study.

CA 5.2.4 Skin irritation

Data Point:	KCA 5.2.4/01
Report Author:	[redacted]
Report Year:	2000
Report Title:	AE C638206 - Rabbit skin irritancy
Report No:	C008137
Document No:	M-197226-01-1
Guideline(s) followed in study:	Directive 92/69/EEC, Part B, Method B.4. (1992); JMAFF 59 NohSan No.4200 (1985); OECD 404 (1992); US-EPA 712-C-98-196. OPPTS 870.2500 (1998)
Deviations from current test guideline:	None
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

The potential of fluopicolide to cause inflammatory or corrosive changes upon first contact with skin was assessed by semi-occluded application of 0.5 g of the test substance to the closely clipped dorsa of three New Zealand White rabbits for four hours. Dermal reactions were assessed at 1, 24, 48 and 72 hours following removal of the dressings.

A single semi-occlusive application of fluopicolide to intact rabbit skin for four hours elicited no dermal irritation in any animal during the study. The mean irritation score (24-72 hours) was 0.0.

In conculsion, fluopicolide was not irritating to rabbit skin according to Regulation (EC) 1272/2008.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 (fluopicolide)
Purity:	97.7% w/w
Batch no.:	PP/241024/2 & PP/241067/1

2. Vehicle and/or positive control

Vehicle:	None

3. Test animals

Species:	Rabbit, females
Strain:	New Zealand White strain
Age:	At least 11 weeks of age
Weight at start:	2.3 to 2.7 kg
Source:	[redacted]
Acclimation period:	Yes

Diet:	Special Diet Services STANRAB (P) SQC pellet
Water:	Water *ad libitum*
Housing:	Individually in stainless steel cages with perforated floors
Temperature:	20.0 to 20.5 °C
Humidity:	26 to 36%
Air changes:	Not given
Photoperiod:	12 hours

B. Study design

1. In-life dates: February 15 to February 18, 2000

2. Animal assignment and treatment

Approximately 24 hours prior to application of the test substance, hair was removed with electric clippers from the dorso-lumbar region of each rabbit, exposing an area of skin approx. 100 mm x 100 mm. Approximately 0.5 g of the test substance was applied under a 2-ply 25 mm x 25 mm gauze pad which had been moistened with 0.5 mL distilled water, to one intact skin site on each animal. Each treatment site was covered with "Elastoplast" elastic adhesive dressing for four hours. The animals were not restrained during the exposure period and were returned to their cages immediately after treatment. At the end of the exposure period, the semi-occlusive dressing and gauze pad were removed, and the treatment site washed with warm water (30 to 40 °C) to remove any residual test substance. The treated area was then blotted dry with absorbent paper.

C. Methods

1. Observations

All animals were observed daily for signs of ill health or toxicity.

2. Dermal observations

Examination of the treated skin was made on Day 1 (i.e. approximately 60 minutes following removal of the dressings) and on Days 2, 3 and 4 (equivalent to approximately 24, 48 and 72 hours after exposure).

Local dermal irritation was assessed using the prescribed numerical system:

Erythema and eschar formation

No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) or eschar formation (injuries in depth) preventing erythema reading	4

Oedema formation:

No oedema	0
Very slight oedema (barely perceptible)	1
Slight oedema (edges of area well-defined by definite raising)	2
Moderate oedema (edges raised approximately 1 mm)	3
Severe oedema (raised more than 1 mm and extending beyond the area of exposure)	4

II. Results and Discussion

A. Results

1. Clinical signs

There were no signs of toxicity or ill health in any rabbit during the observation period.

2. Dermal reactions

The numerical values given to the dermal reactions elicited by fluopicolide are shown in Table 5.2.4- 1.

Table 5.2.4- 1: Dermal reactions

Rabbit no.	E = Erythema O = Oedema	Hours after treatment 1*	24	48	72	Mean irritation score (24-72 hrs)
156 (female)	E	0	0	0	0	0
	O	0	0	0	0	0
157 (female)	E	0	0	0	0	0
	O	0	0	0	0	0
158 (female)	E	0	0	0	0	0
	O	0	0	0	0	0

*: Approximately 1 h after removal of the dressing

A single semi-occlusive application of fluopicolide to intact rabbit skin for four hours elicited no dermal irritation in any animal during the study.

The mean irritation scores (24-72 hours) were 0 for both erythema and oedema.

III. Conclusion

No dermal irritation was observed during the study. The mean irritation score over 24 – 72 hours was 0.0 for both erythema and oedema. Therefore, fluopicolide was not irritating to rabbit skin.

Assessment and conclusion by applicant:

The study was conducted according to OECD TG 404 and is acceptable to determine the skin irritating potential of fluopicolide. Fluopicolide is not a skin irritant under the conditions of this study.

CA 5.2.5 Eye irritation

Data Point:	KCA 5.2.5/01
Report Author:	[redacted]
Report Year:	2000
Report Title:	AE C638206 - Rabbit eye irritancy
Report No:	C008138
Document No:	M-197227-01-1
Guideline(s) followed in study:	Directive 92/69/EEC, Part B, Method B.5. (1992); JMAFF 59 NohSan No.4200 (1985); OECD 405 (1987); US-EPA 712-C-98-195, OPPTS 870.2400 (1998)
Deviations from current test guideline:	Topical and systemic analgesics were not applied as recommended in the current test guideline
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

The potential of fluopicolide to cause damage to the conjunctiva, iris or cornea was assessed in four New Zealand White rabbits. An amount of 0.1 mL of the test substance was instilled into the conjunctival sac of each of the test animals on Day 1. Ocular reactions were assessed at 1, 24, 48 and 72 hours and 7 days after treatment in accordance with the Draize scheme as provided in the OECD guideline No. 405. In one animal (screen animal) the eye was rinsed at 30 seconds post-instillation.

A single instillation of fluopicolide into the rinsed eye of the screen rabbit elicited slight conjunctival irritation at one-hour post-instillation only.

A single instillation of fluopicolide into the unrinsed eye of the rabbit elicited slight conjunctival irritation in all animals from one-hour post instillation. The ocular reactions resolved in all instances within two days following instillation.

In conclusion, fluopicolide was transiently, slightly irritating to the rabbit eye but does not warrant classification according to Regulation (EC) 1272/2008.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 (fluopicolide)
Purity:	97.7% w/w
Batch no.:	PP/241024/2 & PP/241067/1

2. Vehicle and/or positive control

Vehicle:	Not provided

3. Test animals

Species:	Rabbit, females
Strain:	New Zealand White strain
Age:	At least 11 weeks of age
Weight at start:	2.3 to 2.7 kg
Source:	[redacted]
Acclimation period:	Yes
Diet:	Special Diet Services STANRAB (P) SQC pellet
Water:	Water *ad libitum*
Housing:	Individually in stainless steel cages with perforated floors
Temperature:	19.5 to 21 °C
Humidity:	25 to 38%
Air changes:	Not provided
Photoperiod:	12 hours

B. Study design

1. In-life dates: February 29 to March 6, 2000

2. Animal assignment and treatment

The eyes of each animal were examined prior to instillation of the test substance to ensure that there was no pre-existing corneal damage, iridial inflammation or conjunctival irritation.

Screen study - rinsed eye:

One animal was treated in advance of the others, to ensure that, if a severe response was produced, no further animals would be exposed (see Table 5.2.5-2 for screen animal). The treated eye of this animal was rinsed with distilled water 30 seconds after instillation for duration of 30 seconds.

Main study, unrinsed eyes:

One animal was treated in advance of the other two, again to ensure that if a severe response was produced, no further animals would be exposed (see Table 5.2.5-3 for pilot animal). In compliance with the study guideline, the weight of the test substance (which when gently compacted occupied a volume of 0.1 mL) was measured (see Table 5.2.5-1).

Table 5.2.5- 1: Weight of test substance

Syringe	Weight of 1 mL test substance (mg)
1	80
2	99
3	100
4	94
Mean weight of 0.1 mL test substance	93

On all occasions, a volume of 0.1 mL of the test substance (mean weight 93 mg) was placed in the lower everted lid of one eye of each animal. The eyelids were then gently held together for one second before releasing. The contralateral eye remained untreated.

C. Methods

1. Observations

All animals were observed daily for signs of ill health or toxicity.

2. Ocular observations

Examination of the eyes was made after one hour and 1, 2 and 3 days (equivalent to 24, 48 and 72 hours) following instillation. Observation of the eyes was aided by the use of a handheld light.

Ocular irritation was assessed using the prescribed numerical system:

Cornea Opacity: degree of density (area most dense taken for reading):

No ulceration or opacity	0
Scattered or diffuse areas of opacity (other than slight dulling of normal lustre), details of iris clearly visible	1
Easily discernible translucent areas, details of iris slightly obscured	2
Nacreous areas, no details of iris visible, size of pupil barely discernible	3
Opaque cornea, iris not discernible through the opacity	4

Area of cornea involved:

None	0
One quarter (or less) but not zero	1
Greater than one quarter, but less than half	2
Greater than half, but less than three quarters	3
Greater than three quarters, up to whole area	4

Iris:

Normal	0
Markedly deepened rugae, congestion, swelling, moderate circumcorneal hyperaemia or injection, any of these or combination of any thereof, iris still reacting to light (sluggish reaction is positive)	1
No reaction to light, haemorrhage, gross destruction (any or all of these)	2

Conjunctivae Redness (refers to the most severe reading of palpebral and bulbar conjunctivae, as compared to the control eye):

Blood vessels normal	0
Some blood vessels definitely hyperaemic (injected)	1
Diffuse, crimson colour, individual vessels not easily discernible	2
Diffuse beefy red	3

Chemosis (lids and/or nictating membranes):

No swelling	0
Any swelling above normal (includes nictitating membranes)	1
Obvious swelling with partial eversion of lids	2
Swelling with lids about half-closed	3
Swelling with lids more than half-closed	4

Discharge:

No discharge	0
Any amount greater than normal (does not include small amounts observed in inner canthus of normal animals)	1
Discharge with moistening of lids and hairs just adjacent to lids	2
Discharge with moistening of the lids and hairs, and considerable area around the eye	3

II. Results and Discussion

A. Results

1. Clinical signs

There were no signs of toxicity or ill health in any rabbit during the observation period.

2. Ocular reactions

The numerical values given to the ocular reactions elicited by fluopicolide are shown in Table 5.2.5- 2 and Table 5.2.5-3.

Table 5.2.5-2: Screen animal, ocular reactions (rinsed eye)

Readings	Animal	Cornea		Iris	Conjunctiva			Additional findings
		Area involved	Density		Redness	Chemosis	Discharge	
1 h	213	0	0	0	1	0	0	–
24 h		0	0	0	0	0	0	–
48 h		0	0	0	0	0	0	–
72 h		0	0	0	0	0	0	–
Individual 24-48-72 h means		0	0	0	0	0	0	–

Table 5.2.5- 3: Main study, ocular reactions (non-rinsed eyes)

Readings	Animal	Cornea		Iris	Conjunctiva			Additional findings
		Area involved	Density		Redness	Chemosis	Discharge	
1 h	214*	0	0	0	1	0	0	–
	215	0	0	0	1	0	0	
	216	0	0	0	1	0	0	
24 h	214	0	0	0	0	0	0	–
	215	0	0	0	1	0	0	
	216	0	0	0	1	0	0	
48 h	214	0	0	0	0	0	0	–
	215	0	0	0	0	0	0	
	216	0	0	0	0	0	0	–
72 h	214	0	0	0	0	0	0	–
	215	0	0	0	0	0	0	
	216	0	0	0	0	0	0	
Individual 24-48-72 h means	214	0.0	0.0	0.0	0.0	0.0	0.0	–
	215	0.0	0.0	0.0	0.33	0.0	0.0	–
	216	0.0	0.0	0.0	0.33	0.0	0.0	–

* Pilot animal
- Not applicable

Screen study - rinsed eye:

No corneal damage or iris inflammation was seen in the animal.

Injected blood vessels of the conjunctivae were seen in the animal at one-hour post instillation. Reactions had resolved one day after instillation.

Main study - unrinsed eyes:

No corneal damage or iris inflammation was seen in any animal.

Injected blood vessels to a crimson colouration of the conjunctivae were seen in all three animals from one-hour post instillation. Reactions had resolved in all instances two days after instillation.

Mean scores for 24, 48 and 72 hours post instillation were 0.0 for all parameters, with the exception of conjunctival redness, which had a score of 0.33 in two animals.

III. Conclusion

Fluopicolide was transiently slightly irritant to the rabbit eye but does not warrant classification according to Regulation (EC) 1272/2008).

Assessment and conclusion by applicant:

The study was conducted according to OECD TG 405 and is acceptable to determine the eye irritating potential of fluopicolide. Fluopicolide is not an eye irritant under the conditions of this study.

CA 5.2.6 Skin sensitization

Data Point:	KCA 5.2.6/01
Report Author:	[redacted]
Report Year:	2000
Report Title:	Guinea pig skin sensitization study - AE C638206
Report No:	C008139
Document No:	M-197228-01-1
Guideline(s) followed in study:	Directive 96/54/EEC, Part B, Method B.6. (1996); JMAFF 59 NohSan No.4200 (1985); OECD 406 (1992); US-EPA 712-C-98-147, OPPTS 870.2600 (1998)
Deviations from current test guideline:	None
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

The potential of fluopicolide to cause delayed contact hypersensitivity in guinea pigs was assessed by the Magnusson-Kligman maximisation test. Based on the findings of a preliminary study, the closely-clipped dorsa of twenty female Dunkin-Hartley guinea-pigs were subject to intradermal injections on day 1 of Freund's Complete Adjuvant, 10% w/v fluopicolide in sterile water and 10% w/v fluopicolide in a 50:50 mixture of Freund's complete adjuvant in sterile water. Six days later, the same area of skin was treated with a topical application of 100% w/v fluopicolide in sterile water and the test site was covered by an occlusive dressing which was left *in situ* for 48 hours. The same induction procedures were carried out on 10 control animals, with the exception that the test substance was replaced with vehicle in all doses. Two weeks after the topical induction, all animals were challenged by occluded application of 100% fluopicolide in sterile water to the anterior site on the flank and 50% fluopicolide in sterile water to the posterior site on the flank. The occlusive dressings were removed the following day and the condition of the test sites assessed approximately 24 and 48 hours later.

There were no deaths or signs of ill health or toxicity. Body weight changes were similar between control and treated animals.

During the induction phase, necrosis was observed at sites receiving Freund's Complete Adjuvant in all test and control animals following intradermal injections. Slight irritation was seen in six/twenty test animals at sites receiving fluopicolide, 10% w/v in sterile water, whilst no irritation was observed in any control animal receiving sterile water. Following topical application, slight to well-defined erythema was observed in all test animals receiving 100% w/v fluopicolide. Slight erythema was also seen in one control guinea-pig.

The challenge application produced no dermal reactions indicative of skin sensitization in any of the test or control animals. Slight erythema was observed in two test animals at the 24- and 48-hour readings compared with slight to well-defined erythema for two control animals at the 48-hour reading only. The reactions observed were noted to be of similar incidence and severity and as no reactions were observed for any of the remaining test or control animals, the overall response was considered negative.

In conclusion, fluopicolide was not a skin sensitizer in this guinea pig Magnusson and Kligman test.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 (fluopicolide)
Purity:	97.7% w/w
Batch no.:	PP/241024/2 & PP/241067/1

2. Vehicle and/or positive control

Vehicle:	Sterile water

3. Test animals

Species:	Guinea pigs, females
Strain:	Albino guinea pigs of the Dunkin/Hartley strain
Age:	At least 11 weeks of age
Weight at start:	390 – 464 g
Source:	[redacted]
Acclimation period:	Yes
Diet:	Vitamin C enriched guinea-pig diet (Harlan Teklad 9600 FD2 SQC) ad libitum. Hay was given three times each week.
Water:	Water ad lib
Housing:	In groups of five in suspended plastic cages with solid floors and sawdust bedding
Temperature:	18 to 20 °C
Humidity:	43 to 59%
Air changes:	Not given
Photoperiod:	12 hours

B. Study design

1. In-life dates: February 17 to March 20, 2000

2. Animal assignment and treatment

The potential of fluopicolide to cause delayed contact hypersensitivity in guinea pigs was assessed by the Magnusson-Kligman Maximisation Test. Based on the findings of a preliminary study, the closely-clipped dorsa of twenty female Dunkin-Hartley guinea-pigs were subject on day 1 to intradermal injections of Freund's Complete Adjuvant, 10% w/v fluopicolide in sterile water and 10% w/v fluopicolide in a 50:50 mixture of Freund's complete adjuvant in sterile water. Six days later, the same area of skin was treated by topical application of 100% w/v fluopicolide in sterile water and the test site was covered by an occlusive dressing which remained *in situ* for 48 hours. The same induction procedures were carried out on 10 control group animals, with the exception that the test material was replaced by vehicle in all doses. Two weeks after the topical induction, all animals were challenged by occluded application of 100% fluopicolide in sterile water to the anterior site on the flank and 50% fluopicolide in sterile water to the posterior site on the flank. The occlusive dressings were removed the following day and the condition of the test sites assessed approx. 24- and 48-hours later.

C. Methods

1. Observations

All animals were observed daily for signs of ill health or toxicity. The body weight of each guinea-pig of the main study was recorded on Day 1 (day of intradermal injections) and on the last day, during which observations were made of dermal responses to the challenge application.

2. Dermal observations

The dermal reactions resulting from intradermal injection and topical application on the preliminary study, and topical application at the challenge were assessed using the following numerical system.

Erythema and eschar formation:

No erythema	0
Slight erythema	1
Well-defined erythema	2
Moderate erythema	3
Severe erythema (beet redness) to slight eschar formation (injuries in depth)	4

Oedema formation:

No oedema	0
Slight oedema	1
Well-defined oedema (edges of area well-defined by definite raising)	2
Moderate oedema (raised approximately 1 mm)	3
Severe oedema (raised more than 1 millimetre and extending beyond the area of exposure)	4

The approximate diameter (mm) of the dermal response at the intradermal injection sites was recorded in the preliminary study only, in order to assist with the selection of concentrations for the main study.

Any lesions not covered by this scoring system were recorded and described.

The challenge sites were evaluated approx. 24, 48 and 72 hours after removal of the patches. On completion of the study all animals were killed by cervical dislocation.

Dermal reactions in the test animals elicited by the challenge application were compared with the findings simultaneously obtained in the control animals.

A test animal was considered to show positive evidence of delayed contact hypersensitivity if the observed dermal reaction at challenge was clearly more marked and/or persistent than the maximum reaction seen in animals of the control group.

If the dermal reaction seen in a test animal at challenge was slightly more marked and/or persistent than (but not clearly distinguishable from) the maximum reaction seen in control animals, the result for that test animal was classified as inconclusive.

A test animal was considered to show no evidence of delayed contact hypersensitivity if the dermal reaction resulting from the challenge application was the same as, or less marked and/or persistent than the maximum reaction seen in animals of the control group.

II. Results and Discussion

A. Results

1. Clinical signs

There were no signs of toxicity or ill health.

2. Dermal reactions

Preliminary study

In the preliminary study, a dose response relationship for the endpoints of diameter, erythema and oedema was evident from doses ranging from 0.1 – 40% fluopicolide. Based upon these findings, the following concentrations of fluopicolide were selected:

Induction intradermal injection: 10 % fluopicolide w/v in sterile water and 10% w/v fluopicolide in a 50:50 mixture of Freund's complete adjuvant.

This was the highest concentration that caused irritation but did not adversely affect the animals.

Induction topical application: 100% fluopicolide w/v in sterile water.

Topical challenge: 100% w/v and 50% w/v in sterile water.

From preliminary investigations the test material applied topically at 100% w/v did not give rise to any irritating effects.

Main study

Induction

Following intradermal injections, necrosis was recorded at sites receiving Freund´s Complete Adjuvant in all test and control animals.

Slight irritation was seen in 6/20 test animals at sites receiving fluopicolide, 10% w/v in sterile water, whilst no irritation was observed in any control animal receiving sterile water.

Following topical application, slight to well-defined erythema was observed in all test animals receiving 100% w/v fluopicolide. Slight erythema was also seen in one control guinea-pig.

Challenge

The numerical values given to the dermal reactions elicited by the challenge applications are shown in Table 5.6.2- 1.

Slight erythema was observed in two test animals at the 24- and 48-hour reading compared to slight to well-defined erythema for two control animals at the 48-hour reading only. As the reactions observed were of similar incidence and severity and no reactions were observed for any of the remaining test or control animals, all test animals gave negative responses.

Table 5.2.6- 1: Dermal reactions after challenge application with fluopicolide

Guinea pig no.	E=erythema O=Oedema	Score 24 hours A	24 hours P	48 hours A	48 hours P	72 hours A	72 hours P	Results
Freund's treated controls								
815	E	0	0	1	0	0	0	-
	O	0	0	0	0	0	0	
816	E	0	0	0	0	0	0	-
	O	0	0	0	0	0	0	
817	E	0	0	0	0	0	0	-
	O	0	0	0	0	0	0	
818	E	0	0	0	0	0	0	-
	O	0	0	0	0	0	0	
819	E	0	0	0	0	0	0	-
	O	0	0	0	0	0	0	
820	E	0	0	0	0	0	0	-
	O	0	0	0	0	0	0	
821	E	0	0	0	0	0	0	-
	O	0	0	0	0	0	0	
822	E	0	0	0	0	0	0	-
	O	0	0	0	0	0	0	
823	E	0	0	2	0	0	0	-
	O	0	0	0*	0	0	0*	
824	E	0	0	0	0	0	0	-
	O	0	0	0	0	0	0	
Test animals								
825	E	1	0	1	0	0	0	-
	O	0	0	0	0	0*	0	
825	E	0	0	0	0	0	0	-
	O	0	0	0	0	0	0	
826	E	0	0	0	0	0	0	-
	O	0	0	0	0	0	0	
827	E	0	0	0	0	0	0	-
	O	0	0	0	0	0	0	
828	E	0	0	0	0	0	0	-
	O	0	0	0	0	0	0	
829	E	0	0	0	0	0	0	-
	O	0	0	0	0	0	0	
830	E	0	0	0	0	0	0	-
	O	0	0	0	0	0	0	
831	E	0	0	0	0	0	0	-
	O	0	0	0	0	0	0	
832	E	0	0	0	0	0	0	-
	O	0	0	0	0	0	0	
833	E	0	0	0	0	0	0	-
	O	0	0	0	0	0	0	
834	E	1	0	1	0	0	0	-
	O	0	0	0	0	0	0	
835	E	0	0	0	0	0	0	-
	O	0	0	0	0	0	0	
836	E	0	0	0	0	0	0	-
	O	0	0	0	0	0	0	
837	E	0	0	0	0	0	0	-
	O	0	0	0	0	0*	0*	
838	E	0	0	0	0	0	0	-
	O	0	0	0	0	0	0	

Guinea pig no.	E=erythema O=Oedema	Score 24 hours A	24 hours P	48 hours A	48 hours P	72 hours A	72 hours P	Results
839	E	0	0	0	0	0	0	-
	O	0	0	0	0	0	0	
840	E	0	0	0	0	0	0	-
	O	0	0	0*	0*	0*	0*	
841	E	0	0	0	0	0	0	-
	O	0	0	0	0	0	0	
842	E	0	0	0	0	0	0	-
	O	0	0	0	0	0	0	
843	E	0	0	0	0	0	0	-
	O	0	0	0	0	0	0	
844	E	0	0	0	0	0	0	-
	O	0	0	0	0	0	0	

* Dryness and sloughing of the epidermis
A: Anterior site, exposed to fluopicolide, 100 % w/v in sterile water
P: Posterior site, exposed to fluopicolide, 50 % w/v in sterile water
Results: + : Positive; - : Negative; ± : Inconclusive

III. Conclusion

Fluopicolide was not a skin sensitizer in this guinea pig Magnusson and Kligman test.

Assessment and conclusion by applicant:

The study was conducted according to OECD TG 406 and is acceptable to determine the skin sensitising potential of fluopicolide. Fluopicolide is not a skin sensitiser under the conditions of this study.

CA 5.2.7 Phototoxicity

According to the new data requirements (COMMISSION REGULATION (EU) No 283/2013 of 1 March 2013; Official Journal of the European Union, L 93/1, 3.4.2013), the conduct of an *in vitro* phototoxicity study is required "where the active substance absorbs electromagnetic radiation in the range 290-700 nm and is liable to reach the eyes or light-exposed areas of skin, either by direct contact or through systemic distribution. If the Ultraviolet/visible molar extinction/absorption coefficient of the active substance is less than 10 L x mol^{-1} x cm^{-1}, no toxicity testing is required."

For fluopicolide, the absorption maximum is at 203 nm (44519 L/mol*cm)

MEC at 271 nm is 3601 L/mol*cm

MEC at 291 nm is 148 L/mol*cm

MEC at 297 nm is 8.6 L/mol*cm

MEC at 310 nm is 0.69 L/mol*cm

Absorption is above the trigger at 290 nm but declines very rapidly. Generally, OECD TG 432 is considered quite sensitive, and irradiation below 320 nm can be cytotoxic to the cells without any test substance.

According to S10 Photosafety Evaluation of Pharmaceuticals Guidance for Industry (FDA, 2015[11]), direct phototoxicity is considered unlikely if MEC is below 1000 L/mol*cm between 290 and 700 nm.

Considering the rapidly declining absorption at the very end of the relevant range, relevant testing conditions might be difficult to define, and test results might be difficult to interpret due to cytotoxicity.

Therefore, the conduct of a phototoxicity study is not reasonable and considered not to be required.

[11] S10 Photosafety Evaluation of Pharmaceuticals. Guidance for Industry. U.S. Department of Health and Human Services, Food and Drug Administration. ICH, 2015.

CA 5.3 Short-term toxicity

Numerous short-term toxicity studies with fluopicolide have been conducted in mice, rats and dogs at lengths of 4, 13 and 52 weeks. With the exception of a 28-day study by dermal application, all studies were administered via the oral route by dietary administration. The studies are summarized in Table 5.3 -1. The predominant effects were reductions of food consumption and reductions in body weight and/or body weight gain. The liver was identified as target organ in all three species following repeated oral exposure to fluopicolide. In male rats the kidney was additionally affected. Effects on other organs (blood, bones, and adrenals) were only observed at high tested dose levels in the rat sub chronic study. No adverse effects were observed after subacute exposure by the dermal route in rats up to 1,000 mg/kg bw/day.

The effects on the liver observed in rats and mice are considered to be primarily adaptive responses indicating induction of drug metabolizing enzymes in the liver consequent to the extensive hepatic metabolism of fluopicolide. These include increases in relative liver weights and increased incidences and severity of centrilobular hepatocyte hypertrophy. At higher dose levels absolute liver weight was additionally increased and changes in clinical chemical parameters (cholesterol, protein, liver enzymes) were reported indicating an influence on liver function. In one of the sub chronic mice studies an increased incidence of hepatocytic necrosis was observed at ≥ 3,200 ppm. The dog was less sensitive than the rodents and only showed slight liver effects (liver enlargement in single animals, slightly increased liver weights and plasma cholesterol levels) at high dose levels after repeated administration.

Kidney effects were only observed in male rats and characterised by an increase in the severity and incidence of accumulation of hyaline droplets in the proximal tubule, accompanied by single cell death in the proximal tubule epithelium, foci of basophilic (regenerating) tubules, granular casts and increased relative kidney weights at higher dose levels in the sub chronic study only. Hyaline droplets have been recognized as indicator of changes associated with the accumulation of α2µ-globulin. Since the α2µ-globulin is an adult male rat-specific protein it is widely accepted that the renal effects induced in male rats by chemicals causing α2µ-globulin accumulation are unlikely to occur in humans. Therefore, the observed kidney effects are considered non relevant for humans.

Based upon the sub-acute and sub-chronic oral exposure studies with fluopicolide, the lowest NOAEL was derived from the 90-day rat study at 100 ppm (equivalent to 7.4/8.4 mg/kg bw/day (M/F)), based primarily upon an increase in cholesterol plasma levels and increased relative liver and kidney weights with associated histopathological findings.

In the available oral subacute and subchronic studies in rats, mice and dogs, no consistent changes in clinical biochemistry, haematology or urinalysis parameters that indicate severe organ dysfunction were seen in a dose range relevant for STOT-RE classification. Severe organ damage apparent in microscopic examination was only observed at very high doses. According to the CLP guidance (Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures, Version 5.0 July 2017), a 'Specific Target Organ Toxicity – Repeated Exposure (STOT-RE)' classification for fluopicolide is not warranted.

Fluopicolide was discussed at the 53rd meeting of the Committee for Risk Assessment (RAC-53). It was agreed that no classification for STOT RE was warranted in accordance with the criteria outlined in the CLP criteria.

Table 5.3- 1: Summary of short-term toxicity studies

Study	NOAEL	LOAEL	Findings at LOAEL	Reference
Oral route				
Rat 28-day dietary study 0, 20, 200, 2,000 & 20,000 ppm	200 ppm [17.7 mg/kg/d (combined sexes)]	2,000 ppm [179 mg/kg/d (combined sexes)]	↓ bodyweight (F) ↑ water consumption (M) ↑ cholesterol in blood (M/F) ↑ incidence of centrilobular hepatocytic hypertrophy (M/F) ↑ severity of phloxine tartrazine-positive granulation (hyaline droplets) in kidneys (M)	[redacted] 2000; M-199277-01-1 KCA 5.3.1/01
Mouse 28-day dietary study 0, 6, 64, 640 & 6,400 ppm	64 ppm [11.6 mg/kg bw/d (combined sexes)]	640 ppm [115 mg/kg bw/d (combined sexes)]	↑ ALT (M) ↑ rel. liver weight (F) ↑ incidence & severity of hypertrophy of centrilobular hepatocytes (M/F)	[redacted] 2000; M-197343-01-1 KCA 5.3.1/02
Dog 28-day gavage study 0, 10, 100 and 1,000 mg/kg bw/d	1,000 mg/kg bw/d	-	No adverse effects observed	[redacted] 2000; M-197350-01-1 KCA 5.3.1/03
Rat 90-day dietary study 0, 100, 1,400 or 20,000 ppm	100 ppm [7.9 mg/kg bw/d (combined sexes)]	1,400 ppm [114 mg/kg bw/d (combined sexes)]	↑ cholesterol in blood (M) ↑ epithelial cells in urinary sediment (M) ↑ urine volume & ↓ specific gravity (F) ↑ rel. liver weight (M) ↓ abs. and rel. spleen weight (F) ↑ rel. kidney weight (M) ↑ incidence hypertrophy of centrilobular hepatocytes (M) ↑ severity & incidence of trabecular hyperostosis of the bone joint (F) ↑ severity accumulation of hyaline droplets in the proximal kidney tubule and indications of degenerative and regenerative changes in kidney (M)	[redacted] 2000; M-197622-01-1 KCA 5.3.2/01
Mouse 90-day dietary study 0, 50, 200, 800 and 3,200 ppm	50 ppm [10.4 mg/kg bw/d (M)] [12.6 mg/kg bw/d (F)]	200 ppm [37.8 mg/kg bw/d (M)] [52.8 mg/kg bw/d (F)]	↓ cholesterol in blood (M/F)	[redacted] 2006; M-205579-02-1 KCA 5.3.2/02

Study	NOAEL	LOAEL	Findings at LOAEL	Reference
Mouse 90-day dietary study 0, 32, 320, 3,200 and 6,400 ppm	320 ppm [53 mg/kg bw/d (combined sexes)]	3,200 ppm [545 mg/kg bw/d (combined sexes)]	↓ body weight gain (F) ↑ALT (M/F) and AST (M) ↑ abs. & rel. liver weight (M/F) ↑ incidence hypertrophy of centrilobular hepatocytes (M/F) ↑ incidence of hepatocytic necrosis (F)	[redacted] 2000; M-197603-01-1 KCA 5.3.2/05
Dog 90-day gavage study 0, 5, 70 or 1,000 mg/kg bw/d	70 mg/kg bw/d	1,000 mg/kg bw/d	↓ body weight gain (M/F) ↑ abs. & rel. liver weight (M/F)	[redacted] 2000; M-199397-01-1 KCA 5.3.2/03
Dog 52-week gavage study 0, 70, 300 or 1,000 mg/kg/d	300 mg/kg bw/d	1,000 mg/kg bw/d	↓ bodyweight gain (M) ↑ cholesterol in blood (F) ↑ incidence of liver enlargement (M/F)	[redacted] 2002; M-216694-01-1 KCA 5.3.2/04
Dermal route				
Subacute dermal toxicity study in rats 0, 100, 250, 500 & 1,000 mg/kg bw/d	1,000 mg/kg bw/d		No adverse effects observed	[redacted] 2003; M-220782-01-1 KCA 5.3.3/01

M = male F = female

CA 5.3.1 Oral 28-day study

Data Point:	KCA 5.3.1/01
Report Author:	[redacted]
Report Year:	2000
Report Title:	Rat 28-day dietary toxicity study - AE C638206
Report No:	C009846
Document No:	M-199377-01-1
Guideline(s) followed in study:	EEC Commission Directive 92/69/EEC, Annex V, Part 3, Method B7 (1992); OECD 407 (1995); US-EPA OPPTS 870.3100 (1998); JMAFF (1985)
Deviations from current test guideline:	Prostate and seminal vesicles with coagulating glands were not weighed at termination and eye(s), seminal vesicles with coagulating glands and skeletal muscle were not sampled, fixed or examined histopathologically. However, seminal vesicles with coagulating glands were weighed at termination in the rat 2-generation study (Blee, M. A. B.; 2003; M-232532-01-1) at dose levels of up to 2,000 ppm; whilst eyes, seminal vesicles with coagulating glands and skeletal muscle were all sampled, processed and examined histologically in the rat 90 day repeat dose study (Mallyon, B. A.; 2000; M-197622-01-1) at doses up to 20,000 ppm. Therefore, these deviations do not affect the overall acceptability of the study.
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

Groups of 5 male and 5 female Sprague Dawley CRL:(IGS)CDBR rats were administered fluopicolide (batch no., CDB234167-2; purity 99.9%) in the diet at dose levels of 0, 20, 200, 2,000 or 20,000 ppm (corresponding to achieved doses of approximately 1.78, 17.7, 179 and 1770 mg/kg bw/day for the combined sexes) for a minimum of 28 consecutive days.

There were no mortalities or clinical signs of toxicity. The neurotoxicity measurement did not show treatment effects.

At 20,000 ppm, overall body weight gain per day (Day 1-29) was reduced by 32% and 37% compared to control in males and females, respectively. At the end of treatment (Day 29) absolute body weight was reduced in males and females by 14% and 13% compared to control, respectively.

Food consumption for males and females was reduced by 41% and 28% when compared with controls during Week 1 of treatment, whilst food conversion ratios over the treatment period were reduced by 24% and 30%, in males and females, respectively. Water consumption was increased by 27% and 32%, in males and females, respectively when compared with controls.

Biochemical analysis revealed a slight increase in serum cholesterol levels of both sexes, and a slight decrease in ALT in males only. Absolute liver weight was increased by 24% and 13% in males and females, respectively, when compared to controls, and liver weight relative to body weight was increased by 47% in males and by 35% in females.

At necropsy, macroscopic examination revealed enlarged livers in 4/5 males, and both kidneys from 3/5 male animals were pale in colour. Microscopic examination of the liver revealed minimal to slight centrilobular hepatocytic hypertrophy in 5/5 males and 2/5 females at 2,000 ppm and slight to moderate hepatocytic hypertrophy in 5/5 males and 5/5 females at 20,000 ppm. At 200 ppm, the same finding was seen in 2/5 males and 3/5 females. Due to the low incidence and minimal to slight severity at 200 ppm, the findings were considered a non-adverse adaptive change. Additionally, in the kidneys of males, the severity of phloxine tartrazine positive granulation was increased.

In conclusion, the No Observed Effect Level (NOEL) was considered to be 20 ppm, equivalent to 1.78 mg/kg bw/day of technical fluopicolide. The increase in the incidence of centrilobular hepatocyte hypertrophy at 200 ppm was not corroborated by any other indices at this dose level and is considered adaptive. Therefore, the No Observed Adverse Effect Level (NOAEL) is considered to be 200 ppm, equivalent to 17.7 mg/kg bw/day of technical fluopicolide for the combined sexes.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 technical (fluopicolide)
Purity:	99.9% w/w
Batch no.:	CDB234167-2

2. Vehicle and/or positive control

Vehicle:	Diet

3. Test animals

Species:	Rats
Strain:	Sprague Dawley CRL: (IGS) CD BR
Age:	6 weeks of age
Weight at start:	179 g to 222 g for males and 151 g to 192 g for females
Source:	[redacted]
Acclimation period:	Yes
Diet:	Modified SQC Expanded Ground Rat and Mouse Maintenance Diet No. 1 supplied by Special Diet Services Ltd., Stepfield, Witham, Essex, UK
Water:	Water ad lib
Housing:	Caged in groups of five, by sex and dose group
Temperature:	21 ± 2 °C
Humidity:	45 to 65%
Air changes:	Not given
Photoperiod:	12 hours

B. Study design

1. In-life dates: January 19 to February 17, 1999

2. Animal assignment and treatment

The rats were randomized and assigned to the following test groups.

Table 5.3.1- 1: Study design

Group no.	Dose (ppm)	Number of males	Number of females
1	0	5	5
2	20	5	5
3	200	5	5
4	2,000	5	5
5	20,000	5	5

Animals were killed on Study Day 29 (males) and Study Day 30 (females).

3. Diet preparation and analysis

Prior to the start of treatment, a procedure was developed to prepare stable mixtures of the test material in the laboratory rodent diet at the required nominal concentrations.

Test diets were prepared weekly throughout the study. Diet for the highest concentration was prepared using a grinder and a Kenwood mixer with the appropriate quantity of laboratory rodent diet and blended in a Turbula mixer for approximately 20 minutes. Subsequent dietary concentrations were prepared by serial dilution.

Every week, aliquots of each concentration of the freshly prepared test diets were submitted to the Dose Analysis Section in the Toxicology Function of Aventis CropScience UK Ltd. (formally AgrEvo CropScience UK Ltd.), for analysis of the test material concentration. Samples were received in powder form in plastic bags and were stored deep frozen from time of receipt.

The mean results for the test-diet samples, analysed and prepared for dosing, were within the range 92.9% to 100.9% of nominal (acceptable range +10% to -15% of nominal).

Stability was satisfactory over the time of use of the diet (8 days), i.e. % nominal levels declined by a maximum of 7% over 8 days storage at room temperature.

4. Statistics

The significance of differences between control and treated groups was analysed by either parametric or non-parametric statistical tests as appropriate. A maximum 2-tailed probability value of 5% ($p < 0.05$) was considered statistically significant.

The following convention has been used to indicate statistical significance:

* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$

C. Methods

1. Observations

Animals were observed daily for abnormal behaviour, including neuro-muscular coordination and physical appearance. Observations were made once each morning. They were also observed in the afternoon on Mondays to Fridays except on public and Company holidays. More frequent observations were made as necessary. Records of observations included the nature, time of onset, severity and duration of any abnormal behaviour or condition.

2. Body weight and food intake

The weight of the animals was recorded at receipt. Each animal was weighed at randomisation, at the start of treatment, weekly thereafter and at necropsy. For each sex, the amount of food consumed for each cage of animals was measured at weekly intervals throughout the treatment period.

3. Ophthalmoscopic examination

Ophthalmological examinations were conducted by a consultant veterinarian on each animal prior to the start of treatment and on all animals of Groups 1 and 5 just prior to termination. Both eyes of each animal were examined using an indirect ophthalmoscope after instillation of 0.5% w/v Mydriacyl.

4. Neurotoxicity assessments

Prior to the commencement of treatment, on Study Day 2, and weekly thereafter the appearance, behaviour and functional integrity of each animal was assessed using a Functional Observation Battery (FOB). The FOB was comprised of a combination of examinations that assess the reaction of animals to handling on removal from the cage and observations in an open field standard arena. In addition to the FOB, the grip strength of each animal was assessed.

The locomotor behaviour of all animals was measured during Week 4. Activity was monitored using Ethovision (Video tracking, Motion analysis and behaviour recognition system).

The cages were identified by animal number only and the observers were unaware of the experimental group to which the animal belonged. Any deviations from normal were recorded with respect to nature, and where appropriate, incidence or severity.

5. Laboratory investigations (haematology, clinical chemistry, urinalysis)

Blood samples for haematology and for biochemistry were collected on Study Day 28. All samples were obtained from the retro-orbital sinus of each animal, under isoflurane anaesthesia.

The following parameters were determined:

Table 5.3.1- 2: Haematology

Haematocrit (HCT)	Neutrophils (NEUT)
Haemoglobin (HB)	Lymphocytes (LYMP)
Red blood cells (RBC)	Monocytes (MONO)
Mean cell volume (MCV)	Eosinophils (EOS)
Mean cell haemoglobin (MCH)	Basophils (BASO)
Mean cell haemoglobin concentration (MCHC)	Large unstained cells (LUC)
	Reticulocyte count (RET)
Platelets (PLT)	Prothrombin time (PT)
White blood cells (WBC)	Activated partial thromboplastin time (APTT)

Table 5.3.1- 3: Biochemistry

Total protein (PROT)	Glucose (GLUC)
Albumin (ALB)	Total cholesterol (CHOL)
Total globulin (GLOB)	Total bilirubin (TBIL)
A/G ratio (A/G)	Chloride (CL)
Calcium (CA)	Aspartate aminotransferase (AST)
Phosphate (PO4)	Alanine aminotransferase (ALT)
Sodium (NA)	Alkaline phosphatase (AP)
Potassium (K)	G-glutamyl transpeptidase (GGT)
Urea (UREA)	Creatine kinase (CPK)
Creatinine (CREA)	

4. Sacrifice and pathology

Animals were killed by exsanguination via the abdominal aorta under ether anaesthesia. All animals were examined thoroughly, and macroscopic abnormalities were recorded.

The following organs from all animals were weighed at necropsy:

Adrenals	Kidneys	Spleen
Brain	Liver	Testes
Heart	Ovaries	Thymus
Epididymides		

The following organs and tissues from all animals were fixed in 10% neutral buffered formalin:

Adrenals	Lymph nodes (cervical,	Bone (Sternum)
Bone marrow smear	mesenteric)	Stomach
Brain	Nerve (sciatic)	Testes
Caecum	Oesophagus	Thymus
Colon	Ovaries	Thyroid (with parathyroid)
Duodenum	Oviducts	Trachea
Epididymides	Pancreas	Urinary bladder
Heart	Pituitary	Uterus*
Ileum	Prostate	Vagina
Jejunum	Rectum	Any other tissue showing
Kidneys	Spinal cord (3 levels)	macroscopic abnormalities
Liver	Spleen	
Lungs		

* Uterine horns and cervix uteri examined

A bone marrow smear was taken from all animals, except decedants and fixed subsequently at staining. Pinnae were stored with formalin fixed tissues for animal identification.

5. Histopathology

Following fixation, nominal 5 µm sections of all tissues from animals in the control and highest dose groups, the liver from all animals, the kidneys from all male animals and any gross lesions, were prepared and stained with haematoxylin and eosin (except bone marrow smear which was fixed and stained with Wright's stain). In addition, a Cryostat section of the liver from all animals was stained with Oil Red O to demonstrate lipid and sections of the kidneys from all male animals were stained with Phloxine Tartrazine to demonstrate the presence of hyaline droplets.

Tissues were examined for histopathological change with a light microscope by Finn International, One Eyed Lane, Weybread, Diss, Norfolk, UK. The data were entered directly onto a computer terminal using the Roelee Pathology system which then generated the summary tables and individual animal reports.

II. Results and Discussion

A. Results

1. Clinical results

There were no mortalities and no treatment-related clinical signs.

2. Body weight and food and water intake

Body weight

At 20,000 ppm, overall body weight gain per day (Day 1-29) was reduced by 32% and 37% compared to control in males and females, respectively. At the end of treatment (Day 29) absolute body weight was reduced in males and females by 14% and 13% compared to control, respectively.

At 2,000 ppm, in females only, overall body weight gain per day (Day 1-29) was reduced by 30% and absolute body weight at the end of treatment (Day 29) was reduced by 11% compared to control.

At lower doses, body weight development remained unaffected by treatment with fluopicolide.

In general, body weight effects were more marked during the first week of the study.

Table 5.3.1- 4: Mean body weights and body weight gains per day

	Dose level (ppm)				
	0	**20**	**200**	**2,000**	**20,000**
Males					
Body weight [g] *(% difference to control)*					
Day 1	200	205 *(+2.5)*	197 *(-1.5)*	200 *(±0.0)*	198 *(-1.0)*
Day 8	254	261 *(+2.8)*	246 *(-3.1)*	240 *(-5.5)*	207* *(-18.5)*
Day 15	301	317 *(+5.3)*	300 *(-0.3)*	284 *(-5.6)*	250* *(-16.9)*
Day 22	333	358 *(+7.5)*	340 *(+2.1)*	322 *(-3.3)*	286* *(-14.1)*
Day 29	356	391* *(+9.8)*	368 *(+3.4)*	346 *(-2.8)*	305* *(-14.3)*
Body weight gain per day [g] *(% difference to control)*					
Day 1-8	7.8	8.1 *(+3.8)*	6.9 *(-11.5)*	5.8 *(-25.6)*	1.2* *(-84.6)*
Day 8-15	6.6	8.0 *(+21.2)*	7.7 *(+16.7)*	6.3 *(-4.5)*	6.2 *(-6.1)*
Day 15-22	4.6	5.9 *(+28.3)*	5.6 *(+21.7)*	5.4 *(+17.4)*	5.2 *(+13.0)*
Day 22-29	3.3	4.6 *(+39.4)*	4.1 *(+24.2)*	3.4 *(+3.0)*	2.7 *(-18.2)*
Day 1-29[#]	5.6	6.6 *(+17.9)*	6.1 *(+8.9)*	5.2 *(-7.1)*	3.8 *(-32.1)*

	Dose level (ppm)				
	0	**20**	**200**	**2,000**	**20,000**
			Females		
Body weight [g] *(% difference to control)*					
Day 1	174	173 *(-0.6)*	169 *(-2.8)*	170 *(-2.3)*	171 *(-1.7)*
Day 8	206	202 *(-1.9)*	192 *(-6.8)*	192 *(-6.8)*	181 *(-12.1)*
Day 15	230	226 *(-1.7)*	212 *(-7.4)*	208 *(-9.6)*	195 *(-15.2)*
Day 22	247	246 *(-0.4)*	226 *(-8.5)*	225 *(-8.9)*	214 *(-13.7)*
Day 29	257	257 *(±0.0)*	238 *(-7.4)*	228 *(-11.3)*	224 *(-12.8)*
Body weight gain per day [g] *(% difference to control)*					
Day 1-8	4.6	4.2 *(-8.7)*	3.3 *(-28.3)*	3.2 *(-30.4)*	1.4* *(-69.6)*
Day 8-15	3.4	3.5 *(+2.9)*	3.0 *(-11.8)*	2.3 *(-32.4)*	2.0* *(-41.2)*
Day 15-22	2.4	2.7 *(+12.5)*	2.0 *(-16.7)*	2.4 *(±0.0)*	2.7 *(+12.5)*
Day 22-29	1.5	1.6 *(+6.7)*	1.7 *(+33.3)*	0.5 *(-66.7)*	1.5 *(±0.0)*
Day 1-29#	3.0	3.0 *(±0.0)*	2.5 *(-16.7)*	2.1 *(-30.0)*	1.9 *(-36.7)*

* $p < 0.05$; statistically different to controls using Dunnett's test
\# No statistical analysis was performed for overall body weight gain per day (Day 1-29).

<u>Food and water intake</u>

At 20,000 ppm, food consumption during Week 1 of treatment was reduced by 41% and 28% for males and females respectively when compared with the controls. Thereafter, the food intake of treated and control animals was similar. Food conversion ratios over the treatment period were reduced by 24% and 30% in males and females, respectively, when compared with the controls. This change was most apparent during week 1 of treatment, when the ratios were decreased by 27% and 63% for males and females, respectively. At 2,000 ppm, food conversion ratios for females were slightly reduced when compared with controls, particularly during the first two weeks of treatment.

Table 5.3.1- 5: **Mean food consumption (g/animal/day)** *(% difference to control)*

	Dose level (ppm)				
	0	**20**	**200**	**2,000**	**20,000**
			Males		
Week 1	27.0	28.0 *(+3.7)*	25.2 *(-6.7)*	24.0 *(-11.1)*	15.9 *(-41.1)*
Week 2	26.7	28.5 *(+6.7)*	27.7 *(+3.7)*	26.0 *(-2.6)*	24.5 *(-8.2)*
Week 3	25.9	28.6 *(+10.4)*	27.4 *(+5.8)*	27.3 *(+5.4)*	25.6 *(-1.2)*
Week 4	25.0	28.0 *(+12.0)*	26.6 *(+6.4)*	24.7 *(-1.2)*	24.3 *(-2.8)*
			Females		
Week 1	20.0	20.5 *(+2.5)*	18.9 *(-5.5)*	19.5 *(-2.5)*	14.4 *(-28.0)*
Week 2	20.2	20.4 *(+1.0)*	18.7 *(-7.4)*	18.1 *(-10.4)*	20.0 *(-1.0)*
Week 3	20.8	21.6 *(+3.8)*	19.6 *(-5.8)*	20.4 *(-1.9)*	20.0 *(-3.8)*
Week 4	21.1	21.2 *(+0.5)*	20.2 *(-4.3)*	20.1 *(-4.7)*	19.8 *(-6.2)*

Water consumption was measured during Week 3 of the study. At 20,000 ppm, water consumption was increased by 27% and 32%, in males and females, respectively, when compared with the controls. At 2,000 ppm in males only, water consumption was increased by 18% when compared with the controls.

Table 5.3.1- 6: Mean water consumption (g/animal/day) *(% difference to control)*

	Dose level (ppm)				
	0	**20**	**200**	**2,000**	**20,000**
Males					
Week 3	27.2	28.1 *(+3.3)*	28.8 *(+5.9)*	32.2 *(+18.4)*	34.5 *(+26.8)*
Females					
Week 3	25.3	25.4 *(+0.4)*	25.4 *(+0.4)*	24.8 *(-2.0)*	33.3 *(+31.6)*

3. Ophthalmoscopic examinations

There were no treatment-related ophthalmic lesions.

4. Neurotoxicity assessments

No treatment-related effects were observed.

5. Laboratory investigations

Haematology:

There were no treatment-related findings.

Clinical chemistry:

At 20,000 ppm, a slight increase in cholesterol was observed in animals of both sexes, and a slight decrease in alanine aminotransferase was observed in male animals only. At 2,000 ppm, a slight increase in cholesterol was observed in both sexes.

Table 5.3.1- 7: Mean cholesterol and ALT levels in males and females at termination *(% difference to control)*

	Dose level (ppm)				
	0	**20**	**200**	**2,000**	**20,000**
Males					
Cholesterol (mmol/l)	1.61	1.57 *(-2.5)*	2.07* *(+28.6)*	2.42** *(+50.3)*	2.74*** *(+70.2)*
ALT (U/l)	79	75 *(-5.1)*	70 *(-11.4)*	67 *(-15.2)*	54* *(-31.6)*
Females					
Cholesterol (mmol/l)	1.85	2.03 *(+9.7)*	1.93 *(+4.3)*	2.38** *(+28.6)*	2.67*** *(+44.3)*
ALT (U/l)	68	69 *(+1.5)*	75 *(+10.3)*	80 *(+17.6)*	63 *(-7.4)*

* p < 0.05 ; ** p < 0.01 ; *** p < 0.001 statistically different to controls using Bartlett's test

Other statistically significant differences were considered to be of no toxicological significance because their incidence was either not dose-related, or the values were within the normal range of historical controls.

6. Sacrifice and pathology

Macroscopic examination at necropsy revealed enlarged livers at 20,000 ppm in 4/5 males, and both kidneys from 3/5 male animals were pale in colour. At 2,000 ppm, both kidneys of 3/5 male animals were recorded as pale.

At 20,000 ppm, absolute liver weight was increased by 24% and 13% in males and females, respectively, when compared to the controls. Liver weight relative to body weight was increased by 47% in males and by 35% in females, compared to the controls.

Table 5.3.1- 8: Liver weights in males and females following 4-week treatment period *(% difference to control)*

	Dose level (ppm)				
	0	**20**	**200**	**2,000**	**20,000**
Males					
Terminal body weight (g)	352.2	385.4 *(+9.4)*	363.5 *(+3.2)*	337.6 *(-4.1)*	297.7** *(-15.5)*
Liver weight (g)	12.78	13.89 *(+8.7)*	13.82 *(+8.1)*	14.12 *(+10.5)*	15.91* *(+24.5)*
Liver relative to bodyweight	3.63	3.60 *(-0.8)*	3.78 *(+4.1)*	4.18 *(+15.2)*	5.35** *(+47.4)*
Females					
Terminal body weight (g)	260.0	258.4 *(-0.6)*	236.9 *(-8.9)*	233.2 *(-10.3)*	217.7 *(-16.3)*
Liver weight (g)	9.58	9.40 *(-1.9)*	8.52 *(-11.1)*	9.23 *(-3.7)*	10.79 *(+12.6)*
Liver relative to bodyweight	3.67	3.61 *(-1.6)*	3.59 *(-2.2)*	3.95 *(+7.6)*	4.95** *(+34.9)*

* p < 0.05 ; ** p < 0.01 ; statistically different to controls using Bartlett's test

7. Histopathology

At 20,000 ppm, centrilobular hepatocytic hypertrophy was recorded for all males and females. This finding was recorded as slight in all females and slight for 3/5 males and moderate for 2/5 males. At 2,000 ppm, all males and 2/5 females were observed to have centrilobular hepatocytic hypertrophy. This was recorded as minimal in both females and 1/5 males and slight for 4/5 males. At 200 ppm, centrilobular hepatocytic hypertrophy was seen in 2/5 males (one animal scored as minimal and the other as slight) and in 3/5 females (minimal).

Table 5.3.1- 9: Centrilobular hepatocyte hypertrophy – Number of animals affected

Severity	**Dose (ppm)**				
	0	**20**	**200**	**2,000**	**20,000**
Males					
Minimal	0	0	1	1	0
Slight	0	0	1	4	3
Moderate	0	0	0	0	2
Total	**0**	**0**	**2**	**5**	**5**
Females					
Minimal	0	0	3	2	0
Slight	0	0	0	0	5
Total	**0**	**0**	**3**	**2**	**5**

At 20,000 ppm and 2,000 ppm, the severity of phloxine tartrazine positive granulation in the renal sections of male animals was increased from minimal to moderate/severe. Hyaline droplets have been recognized as indicator of changes associated with the accumulation of α2μ-globulin. Since the α2μ-globulin is an adult male rat-specific protein it is widely accepted that the renal effects induced in male rats by chemicals causing α2μ-globulin accumulation are unlikely to occur in humans[12].

Table 5.3.1- 10: Kidney: Phloxine tartrazine-positive granulation in males – Number of animals affected

Severity	Dose (ppm)				
	0	20	200	2,000	20,000
Males					
Minimal	4	2	3	0	0
Slight	0	2	2	0	1
Moderate	1	0	0	3	3
Severe	0	0	0	2	1
Total	**5**	**4**	**5**	**5**	**5**

III. Conclusion

The NOAEL in the 28-dietary study in rats with fluopicolide was considered to be 200 ppm (equivalent to 17.8 mg/kg bw/day fluopicolide) for both sexes based on reduction in body weight gain in females, increased levels of cholesterol (suggestive of impaired liver function), increase in the absolute and relative liver weights in males, increased incidence and/or severity of centrilobular hepatocyte hypertrophy in both sexes and increased incidence and severity of phloxine tartrazine granulation at dose levels of ≥ 2,000 ppm (equivalent 179 mg/kg bw/day). The target organs for toxicity were the liver and kidney. The increase in the incidence of centrilobular hepatocyte hypertrophy at 200 ppm was not corroborated by any other indices at this dose level and is considered adaptive.

Assessment and conclusion by applicant:

The study was conducted according to OECD TG 407 and is acceptable to determine the short-term oral toxicity of fluopicolide in the rat. A NOAEL of 200 ppm (equivalent to 17.8 mg/kg bw/d in males and females) was determined from this study.

[12] Hard GC, Rodgers IS, Baetcke KP, Richards WL, McGaughy RE, Valcovic LR. Hazard evaluation of chemicals that cause accumulation of alpha 2μ-globulin, hyaline droplet nephropathy, and tubule neoplasia in the kidneys of male rats. Environ Health Perspect. 1993 Mar;99:313-49.

Data Point:	KCA 5.3.1/02
Report Author:	[redacted]
Report Year:	2000
Report Title:	Mouse 28-day dietary toxicity study - AE C638206
Report No:	C008274
Document No:	M-197343-01-1
Guideline(s) followed in study:	OECD 407 (1995); EEC Commission Directive 92/69/EEC, Annex V, Part 3, Method B7 ; US-EPA OPPTS 870.3100 (1998); JMAFF 1985)
Deviations from current test guideline:	Method: Deviations from current guideline SANCO/3029/99 rev. 4: There is no linearity data presented and the accuracy and precision data are determined from the procedural recoveries in the toxicological studies. However, there are at least 14 recoveries at the LOQ level and at the highest fortification level with mean accuracies between 70-110% and RSD < 20%. Considering that this analytical method is validated in support of toxicological cold studies, the method validation is considered fit for purpose. Study: Deviations from the current OECD guideline (407, 2008): - Prostate and seminal vesicles with coagulating glands were not weighed at termination - Eye(s), seminal vesicles with coagulating glands and skeletal muscle were not sampled, fixed or examined histopathologically Although performed in the rat, rather than the mouse, eyes, seminal vesicles and skeletal muscle were all sampled, processed and examined histologically in the rat 90- day repeat dose study (Wason, S. M.; 2006; M-205579-02-1) at doses up to 3,200 ppm. Therefore these deviations do not affect the overall acceptability of the study.
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

Groups of 5 male and 5 female CD-1 mice were fed diet containing 0, 6, 64, 640 or 6,400 ppm of technical fluopicolide (equivalent to 1.07, 11.6, 115 and 1,111 mg/kg bw/day for the combined sexes) for a minimum of 28 consecutive days. At 6,400 ppm, biochemical analyses revealed a slight increase in alanine aminotransferase and alkaline phosphatase activities in male animals only. Absolute liver weight was increased by 33% and 50%, in males and females, respectively, when compared with the controls. Liver weight relative to body weight was also increased by 42% and 58%, in males and females, respectively, when compared with the controls. Histopathological examination of the liver indicated an increase in incidence and severity of hypertrophy of centrilobular hepatocytes. At 640 ppm, there was a slight increase in alanine aminotransferase activity for males and absolute and relative liver weights were increased in females only by 19% when compared with the controls. In addition, there was an increase in severity of hypertrophy of centrilobular hepatocytes in males and females. There were no treatment-related effects at 64 and 6 ppm.

The NOAEL in the 28-dietary study in mice was 64 ppm, (equivalent to 10.4 and 12.9 mg/kg bw/day, in males and females, respectively) based on increased liver weight and centrilobular hepatocyte hypertrophy at dose levels of ≥640 ppm (equivalent 100 and 129 mg/kg bw/day in male and female rats respectively).

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 technical (fluopicolide)
Purity:	99.0% w/w
Batch no.:	CDB234187-1

2. Vehicle and/or positive control

Vehicle:	Diet

3. Test animals

Species:	Mice
Strain:	CRLCD-1 (ICR)BR
Age:	35 days of age
Weight at start:	27.6 g to 33.9 g for males and 21.4 g to 26.6 g for females
Source:	[redacted]
Acclimation period:	Yes
Diet:	Modified SQC Expanded Ground Rat and Mouse Maintenance Diet No. 1 supplied by Special Diet Services Ltd., Stepfield, Witham, Essex, UK
Water:	Water ad lib
Housing:	Housed in groups of five, by sex and dose group
Temperature:	21 ± 2 °C
Humidity:	45 to 65%
Air changes:	Not given
Photoperiod:	12 hours

B. Study design

1. In-life dates: March 1 to April 8, 1999

2. Animal assignment and treatment

The mice were randomized and assigned to the following test groups.

Table 5.3.1- 11: Study design

Group no.	Dose (ppm)	Number of males	Number of females
1	0	5	5
2	6	5	5
3	64	5	5
4	640	5	5
5	6,400	5	5

Groups of 5 male and 5 female CRLCD-1 (ICR) BR mice were administered in the diet fluopicolide (batch N° CDB234187-1, purity 99.0%) at nominal concentrations of 0 (control), 6, 64, 640 or 6,400 ppm (equivalent 0, 0.98, 10.4, 100 and 980 mg/kg bw/day in males and 0, 1.19, 12.9, 129 and 1,242 mg/kg bw/day in females) for a minimum of 28 consecutive days. The animals were killed on Study Day 29 (males) and Study Day 30 (females).

3. Diet preparation and analysis

The test diet was prepared prior to the study with the appropriate quantity of test material in the diet using a Turbula T50 automatic mixer to produce diet containing the highest concentration. Subsequent dietary concentrations were prepared by serial dilution. Prior to administration, the study mixes were analysed to demonstrate that homogeneity and stability of the test material in the laboratory rodent diet were at acceptable levels for all required nominal concentrations.

Aliquots of each concentration of the test diets were submitted for analysis of the test material concentration. Samples were received in powder form in plastic bags and were stored deep frozen from time of receipt.

The mean results for the test diet samples analysed and prepared for dosing were within the range 90.5% to 101.0% of nominal (acceptable range +10% to -15% of nominal).

Homogeneity was shown to be satisfactory at all levels, i.e. mean values obtained for top, middle and bottom samples were within the acceptable range 90% to 110% of nominal and these mean % nominal values differed by < 10%.

Stability was satisfactory over the time of use of the diet (8 days), i.e. % nominal levels declined by a maximum of 12% over 8 days storage at room temperature.

4. Statistics

The significance of differences between control and treated groups was analysed by either parametric or non-parametric statistical tests as appropriate. A maximum 2-tailed probability value of 5% ($p < 0.05$) was considered statistically significant.

The following convention has been used to indicate statistical significance:

* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$

C. Methods

1. Observations

Animals were observed daily for abnormal behaviour, including neuro-muscular coordination and physical appearance. Observations were made each morning. Animals were also observed in the afternoon on Mondays to Fridays, except on public and company holidays. More frequent observations were made as necessary. Records of observations included the nature, time of onset, severity and duration of any abnormal clinical sign/behaviour.

2. Body weight and food intake

Each animal was weighed upon receipt, at randomisation, at the start of treatment, at weekly intervals thereafter and at termination.

For each sex, the amount of food consumed for each cage of animals was measured at weekly intervals throughout the treatment period.

3. Ophthalmoscopic examination

Not conducted in mice.

4. Laboratory investigations (haematology, clinical chemistry, urinalysis)

Samples for haematology were collected on Study Day 23 whilst samples for biochemistry were collected on the day of necropsy. All samples were obtained from the retro-orbital sinus under isoflurane anaesthesia.

The parameters listed below were measured or derived.

Table 5.3.1- 12: Haematology

Haematocrit (HCT)	White blood cells (WBC)
Haemoglobin (HB)	Neutrophils (NEUT)
Red blood cells (RBC)	Lymphocytes (LYMP)
Mean cell volume (MCV)	Monocytes (MONO)
Mean cell haemoglobin (MCH)	Eosinophils (EOS)
Mean cell haemoglobin concentration (MCHC)	Basophils (BASO)
	Large unstained cells (LUC)
Platelets (PLT)	Reticulocytes (RET)

Table 5.3.1- 13: Biochemistry

Total protein (PROT)	Glucose (GLUC)
Albumin (ALB)	Total cholesterol (CHOL)
Total globulin (GLOB)	Total bilirubin (TBIL)
A/G ratio (A/G)	Chloride (CL)
Calcium (CA)	Aspartate aminotransferase (AST)
Phosphate (PO4)	Alanine aminotransferase (ALT)
Sodium (NA)	Alkaline phosphatase (AP)
Potassium (K)	G-glutamyl transpeptidase (GGT)
Urea (UREA)	Creatine kinase (CPK)
Creatinine (CREA)	

5. Sacrifice and pathology

Animals were killed by exsanguination via the abdominal aorta under ether anaesthesia. All animals were examined thoroughly and macroscopic abnormalities recorded.

The following organs from all animals were weighed at necropsy:

Table 5.3.1- 14: Organ weights

Brain	Adrenals	Spleen
Kidneys	Liver	Epididymides
Ovaries	Thymus	
Heart	Testes	

The following organs and tissues from all animals were fixed in 10% neutral buffered formalin.

Adrenals	Lungs (inflated)	Testes
Bone - sternum	Mesenteric l. node	Thymus
Brain	Nerve (sciatic)	Thyroid (with
Caecum	Oesophagus	parathyroid)
Cervical L. node	Ovaries	Trachea
Colon	Oviducts	Urinary Bladder
Duodenum	Pancreas	Uterus*
Epididymides	Pituitary	Vagina
Heart	Prostate	Any other tissue showing
Ileum	Rectum	macroscopic abnormalities
Jejunum	Spleen	
Kidneys	Spinal cord	
Liver	Stomach	

*Uterine horns and cervix examined.

A bone marrow smear was taken from all animals and fixed subsequently at staining. Pinnae were stored with formalin fixed tissues for animal identification.

6. Histopathology

Following fixation, all tissues from animals in the control and highest dose groups (with the exception of pinnae), and sections of the liver from all intermediate dose groups, were prepared and stained with haematoxylin and eosin (except bone marrow smear which was fixed and stained with Wright's stain). An additional cryostat section of liver was stained with Oil red O to demonstrate lipid from all dose groups. After at least three weeks in fixative, the brain and spinal cord from all animals were processed to wax block. Tissues were examined for histopathological change with a light microscope by Peter Finn of Finn International, One Eyed Lane, Weybread, Diss, Norfolk, IP21 5TT, UK. The data were entered directly into a computer terminal using the Roelec Pathology system which then generated the summary tables and individual animal reports.

II. Results and Discussion

A. Results

1. Clinical results

There were no mortalities and no treatment-related clinical signs.

2. Body weight and food intake

Body weight

There were no treatment-related effects on body weight.

Table 5.3.1- 15: Mean body weights (g)

	Dose level (ppm)								
	0	6	%[a]	64	%[a]	640	%[a]	6,400	%[a]
Males									
Day 1	31.1	30.1	*(-3.2)*	29.8	*(-4.2)*	30.2	*(-2.9)*	31.5	*(+1.3)*
Day 8	34.7	33.4	*(-3.7)*	33.5	*(-3.5)*	32.6	*(-6.1)*	33.7	*(-2.9)*
Day 15	37.5	34.6	*(-7.7)*	35.7	*(-4.8)*	34.2	*(-8.8)*	35.0	*(-6.7)*
Day 22	39.2	35.9	*(-8.4)*	37.3	*(-4.8)*	35.8	*(-8.7)*	37.1	*(-5.4)*
Day 29	40.1	35.6	*(-11.2)*	35.8*	*(-10.7)*	36.3	*(-9.5)*	37.9	*(-5.5)*
Females									
Day 1	23.0	23.4	*(+1.7)*	23.4	*(+1.7)*	22.8	*(-0.9)*	23.7	*(+3.0)*
Day 8	25.1	25.7	*(+2.4)*	26.6	*(+6.0)*	25.3	*(+0.8)*	24.6	*(-2.0)*
Day 15	26.3	26.8	*(+1.9)*	26.4	*(+0.4)*	26.3	*(±0.0)*	25.7	*(-2.3)*
Day 22	28.1	27.8	*(-1.1)*	28.9	*(+2.8)*	27.7	*(-1.4)*	26.3	*(-6.4)*
Day 29	28.2	27.7	*(-1.8)*	28.8	*(+2.1)*	28.3	*(+0.4)*	26.0	*(-7.8)*

[a] *% difference compared to control*
* $p < 0.05$; statistically different to controls using Dunnett´s test

Food intake

There were no treatment-related effects on food consumption. Food conversion ratios were reduced in most of the treatment groups. However, food conversion ratios were highly variable, and no consistent dose-dependent effect was observed.

Table 5.3.1- 16: Mean food consumption (g/animal/day)

	Dose level (ppm)								
	0	6	%[a]	64	%[a]	640	%[a]	6,400	%[a]
Males									
Week 1	5.7	5.8	*(+1.8)*	5.8	*(+1.8)*	5.5	*(-3.5)*	5.1	*(-10.5)*
Week 2	5.8	5.6	*(-3.4)*	5.9	*(+1.7)*	5.3	*(-8.6)*	5.4	*(-6.9)*
Week 3	5.9	5.6	*(-5.1)*	5.8	*(-1.7)*	5.6	*(-5.1)*	5.9	*(±0.0)*
Week 4	5.7	5.1	*(-10.5)*	5.5	*(-3.5)*	5.3	*(-7.0)*	5.6	*(-1.8)*
Females									
Week 1	5.1	5.4	*(+5.9)*	5.3	*(+3.9)*	4.9	*(-3.9)*	4.9	*(-3.9)*
Week 2	5.1	5.3	*(+3.9)*	5.4	*(+5.9)*	5.3	*(+3.9)*	4.8	*(-9.8)*
Week 3	5.2	5.2	*(±0.0)*	5.9	*(+13.5)*	5.9	*(+13.5)*	5.3)	*(+1.9)*
Week 4	5.1	5.5	*(+7.8)*	5.6	*(+9.8)*	5.6	*(+9.8)*	4.9	*(-3.9)*

[a] *% difference compared to control*

Table 5.3.1- 17: Mean food conversion (%)

	Dose level (ppm)				
	0	**6**	**64**	**640**	**6,400**
Males					
Week 1	8.9	8.2	9.2	6.2	6.3
Week 2	6.8	2.9	5.2	4.3	3.4
Week 3	4.3	3.4	4.0	4.0	5.1
Week 4	2.0	-0.9	-3.9	1.5	2.1
Mean (week 1-4) *(% difference to control)*	**5.5**	**3.4** *(-38.2)*	**3.6** *(-34.6)*	**4.0** *(-27.3)*	**4.2** *(-23.6)*
Females					
Week 1	6.0	6.2	8.6	7.2	2.8
Week 2	3.3	2.7	-0.5	2.8	3.0
Week 3	4.8	2.8	6.0	3.4	1.7
Week 4	0.3	-0.1	-0.3	1.3	-0.9
Mean (week 1-4) *(% difference to control)*	**3.6**	**2.9** *(-19.4)*	**3.5** *(-2.8)*	**3.7** *(+2.8)*	**1.7** *(-52.8)*

3. Ophthalmoscopic examinations

Not performed.

4. Laboratory investigations

Haematology:

There were no treatment-related findings.

Clinical chemistry:

At 640 and 6,400 ppm, a slight increase in alanine aminotransferase was observed. At 6,400 ppm, alkaline phosphatase was slightly and statistically non-significantly increased in males only.

Any other statistically significant differences were considered not to be treatment-related because their incidence was either not dose-related or the values were within the normal range of historical control data.

Table 5.3.1- 18: Clinical chemistry

Parameter	Dose level (ppm)								
	0	**6**	*%[a]*	**64**	*%[a]*	**640**	*%[a]*	**6,400**	*%[a]*
Males									
ALT (U/L)	42	41	*(+2.4)*	46	*(+9.5)*	76**	*(+81.0)*	104**	*(+147.6)*
ALP (U/L)	58	71	*(+22.4)*	66	*(+13.8)*	81	*(+39.7)*	134	*(+131.0)*
Females									
ALT (U/L)	35	31	*(-11.4)*	35	*(±0.0)*	52**	*(+48.6)*	54**	*(+54.3)*
ALP (U/L)	98	80	*(-18.4)*	97	*(-1.0)*	87	*(-11.2)*	88	*(-10.2)*

[a] *% difference compared to control*

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

5. Sacrifice, pathology

There were no treatment-related findings at necropsy.

6. Organ weights

At 6,400 ppm, absolute liver weight was increased by 33% and 50%, in males and females, respectively when compared with controls. Liver weight relative to body weight was also increased by 42% and 58%, in males and females, respectively, when compared with controls.

At 640 ppm, liver absolute and body weight relative liver weights were increased in females only by 19%, when compared with the controls.

There were no other treatment-related effects on organ weights.

Table 5.3.1- 19: Liver weights in males and females

	Dose level (ppm)								
	0	**6**	**%[a]**	**64**	**%[a]**	**640**	**%[a]**	**6,400**	**%[a]**
Males									
Terminal body weight (g)	38.2	33.6*	*(-12.0)*	33.9	*(-11.3)*	34.8	*(-8.9)*	35.8	*(-6.3)*
Liver weight (g)	1.91	1.69	*(-11.5)*	1.73	*(-9.4)*	1.94	*(+1.6)*	2.54**	*(+33.0)*
Liver relative to bodyweight	5.01	5.02	*(+0.2)*	5.13	*(+2.4)*	5.60	*(+11.8)*	7.11**	*(+41.9)*
Females									
Terminal body weight (g)	26.9	27.0	*(+0.4)*	27.4	*(+1.9)*	27.0	*(+0.4)*	25.7	*(-4.5)*
Liver weight (g)	1.33	1.32	*(-0.8)*	1.52	*(+14.3)*	1.58	*(+18.8)*	2.00**	*(+50.4)*
Liver relative to bodyweight	4.92	4.88	*(+0.8)*	5.50	*(+11.8)*	5.86**	*(+19.1)*	7.77**	*(+57.9)*

[a] *% difference compared to control*

* $p < 0.05$; ** $p < 0.01$, statistically different to controls using Dunnett's test

7. Histopathology

At 6,400 ppm, there was an increase in the incidence (5/5 males and 5/5 females) and severity (mainly moderate; 4/5 males, 5/5 females) of hypertrophy of centrilobular hepatocytes in the liver compared to control animals. At 640 ppm, there was also an increase in the incidence (5/5 males and 4/5 females) and severity (mainly slight; 3/5 animals each sex) of hypertrophy of centrilobular hepatocytes in the liver.

At the high dose level of 6,400 ppm, the animals remained unaffected by treatment with fluopicolide regarding the incidence of focal coagulative necrosis in the liver. At 640 ppm, slight focal coagulative necrosis was observed in 1/5 females. At 64 ppm, only minimal focal coagulative necrosis was seen in 1/5 males.

The finding of focal coagulative necrosis in the liver was not considered treatment-related given the low incidence, lack of dose response and the missing evidence of progression of severity with increasing dose levels.

Table 5.3.1- 20: Selected histopathological liver findings – Number of animals affected

Finding / Severity	Dose level (ppm)				
	0	**6**	**64**	**640**	**6,400**
Centrilobular hepatocyte hypertrophy					
Males					
Minimal	3	3	3	1	0
Slight	0	0	1	3	1
Moderate	0	0	0	1	4
Total	**3**	**3**	**4**	**5**	**5**
Females					
Minimal	1	3	0	1	0
Slight	0	0	0	3	0
Moderate	0	0	0	0	5
Total	**1**	**3**	**0**	**4**	**5**
Focal coagulative necrosis					
Males					
Minimal	0	0	1	0	0
Slight	0	0	0	0	0
Total	**0**	**0**	**1**	**0**	**0**
Females					
Minimal	0	0	0	0	0
Slight	0	0	0	1	0
Total	**0**	**0**	**0**	**1**	**0**

III. Conclusion

The main target organ in mice was the liver. The NOAEL in the 28-dietary study in mice was 64 ppm, (equivalent to 10.4 and 12.9 mg/kg bw/day, in males and females, respectively) based on increased liver weight and centrilobular hepatocyte hypertrophy at dose levels of ≥ 640 ppm (equivalent 100 and 129 mg/kg bw/day in male and female mice respectively).

Assessment and conclusion by applicant:
The study was conducted in accordance with OECD TG 407 and is acceptable to determine the short-term oral toxicity of fluopicolide in mice. A NOAEL of 64 ppm (equivalent to 10.4 and 12.9 mg/kg bw/d in males and females) was determined from this study.

Data Point:	KCA 5.3.1/03
Report Author:	[redacted]
Report Year:	2000
Report Title:	Dog 28-day oral toxicity study Code: AE C638206 00 1C99 0005
Report No:	C008283
Document No:	M-197350-01-1
Guideline(s) followed in study:	OECD 409 (1998); EEC, Commission Directive 92/69/EEC, Annex V, Part B, Method B7 (1992); JMAFF (1985) US-EPA OPPTS 870.3150 (1998)
Deviations from current test guideline:	Method: Deviations from current guideline SANCO/3029/99 rev. 4: There is no linearity data presented and the accuracy and precision data are determined from the test suspension samples in the toxicological study. However, there are at least 6 samples per concentration level with mean accuracies between 70-110% and RSD < 20%. Considering that this analytical method is validated in support of a toxicological cold study, the method validation is considered fit for purpose. ;Study: This is a 28-day study, serving primarily as a dose range finding (DRF) study for a subsequently conducted 90-day study performed according to OECD TG 409 (1998). As such, it is considered that the study was performed to the principles of OECD 409 (1998) with appropriate adjustments for a study of this type – i.e. a DRF. Therefore, listing deviations is not considered appropriate.
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

Groups of 2 male and 2 female beagle dogs were treated by gavage, at dose levels of 0, 10, 100 and 1,000 mg/kg bw/day of technical fluopicolide, for at least 28 consecutive days. The test substance was administered in a vehicle of 1.0% w/v methyl cellulose in distilled water at a constant volume of 5 mL/kg bw. Controls received the vehicle alone. There were no mortalities or clinical signs of toxicity.

At 1,000 mg/kg bw/day, biochemistry analyses revealed a slight increase in cholesterol concentration on Study Days 14 and 29 for one male (number 947). This animal also had an increase in absolute liver weight and liver weight relative to body weight of 34% and 44%, respectively, when compared to controls. There were no treatment-related effects for males at 100 and 10 mg/kg bw/day or treated females.

In the absence of histopathological correlates and based upon the fact that the minor hepatic changes (slight increase in cholesterol and increased absolute and relative liver weight) at 1,000 mg/kg bw/day were observed in a single male only, it is considered that the NOAEL under the conditions of this study was 1,000 mg/kg bw/day.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 technical (fluopicolide)
Purity:	96.9% w/w
Batch no.:	T/035/99

2. Vehicle and/or positive control

Vehicle:	1.0% w/v methyl cellulose in distilled water

3. Test animals

Species:	Dogs
Strain:	Beagle
Age:	6 – 7 months old
Weight at start:	10.5 to 11.9 kg for males, and from 8.3 to 9.8 kg for females
Source:	[redacted]
Acclimation period:	Yes
Diet:	Modified SQC Expanded Pellet Laboratory Diet A, supplied by Special Diet Services Ltd., Stepfield, Witham, Essex, UK
Water:	Water ad lib. through automatic valves
Housing:	The dogs were housed in pairs (except during feeding and dosing when they were housed individually), by sex and dose group, under controlled environmental conditions, in a solid floor pen, measuring approximately 1 m x 4.5 m, with wood chips as bedding
Temperature:	18 ± 2 °C
Humidity:	45 to 65%
Air changes:	Not given
Photoperiod:	12 hours

B. Study design

1. In-life dates: June 10 to August 5, 1999

2. Animal assignment and treatment

The dogs were randomized and assigned to the following test groups.

Table 5.3.1- 21: Study design

Group no.	Dose (mg/kg bw/day)	Number of males	Number of females
1	0	2	2
2	10	2	2
3	100	2	2
4	1,000	2	2

Dose levels were 0, 10, 100 and 1,000 mg/kg bw/day. The test substance was suspended in 1.0% w/v methyl cellulose in distilled water, and administered at a constant volume of 5 mL/kg bw. Males were treated for 29 consecutive days, females for 30 consecutive days.

3. Preparation and analysis of dosing suspensions

Prior to the start of treatment, a procedure was developed to reliably prepare homogeneous mixtures of the test material in the vehicle, 1.0% w/v methyl cellulose in distilled water, at the required nominal concentrations of 2, 20 and 200 mg/mL (equivalent to 10, 100 and 1000 mg/kg bw/day dose levels, respectively).

Throughout the study dosing suspensions were generally prepared one day in advance of dosing, except for suspensions dosed over the weekend and on Monday, which were made up on the preceding Friday.

On each occasion, the required volume of dosing suspension was prepared by gradually adding the vehicle to the appropriate quantity of test material and thoroughly mixing them. Initially, a smooth paste was prepared which was made up to volume with more vehicle and the mixture was then homogenised.

On one occasion every week, aliquots of each concentration of the freshly prepared dosing suspensions were submitted to the Dose Analysis Section of Aventis CropScience UK Limited (formerly AgrEvo UK Limited, Chesterford Park, for analysis of test material concentration.

Homogeneity and stability were analysed at all dose levels from the trial mix samples stored at room temperature for 0, 1, 2 and 4 days. The study mix samples from Days 1 and 22 were analysed at all dose levels.

The mean results for the test suspension samples analysed were within the range of 82.9% to 102.8% of nominal (acceptable range +20% to -20% of nominal).

Homogeneity was shown to be satisfactory in the trial mix, i.e. mean values were within the acceptable range of 80% to 120% of nominal, and standard deviation values were less than 10%.

Re-analysis of the trial mix after storage at 4 °C for 0, 1, 2 and 4 days indicated that the suspensions were stable over 4 days at this temperature.

4. Statistics

Not conducted – only 2 animals per sex per dose group.

C. Methods

1. Observations

Each dog was given a thorough clinical examination prior to the start of and at the end of the treatment period. Animals were observed daily for abnormal behaviour, including neuro-muscular coordination and physical appearance. Observations were made once each morning. They were also observed in the afternoon on Mondays to Fridays except on public and Company holidays. More frequent observations were made as necessary. Records of observations included the nature, time of onset, severity and duration of any abnormal clinical sign/behaviour.

2. Body weight and food intake

The weight of each dog was recorded upon receipt at randomisation, on Study Days -14 and -7, and at the start of treatment. Thereafter, each animal was weighed weekly throughout the treatment period and at necropsy.

Food consumption for each animal was measured daily for both 1 week prior to the start of treatment, and throughout the treatment period.

3. Ophthalmoscopic examination

Ophthalmological examinations were conducted by a consultant veterinarian on each dog prior to the start of treatment, and during the last week of treatment. Both eyes of each animal were examined using an indirect ophthalmoscope after instillation of 1.0% w/v Mydriacyl.

4. Laboratory investigations (Haematology, clinical chemistry, urinalysis)

Blood samples for haematology and biochemistry were collected from the jugular vein prior to treatment (Study Days -14 and -1) and on Days 14 and 29 of treatment.

Samples for urinalysis were collected directly from the bladder at necropsy.

The parameters listed below were measured or derived:

Table 5.3.1- 22: Haematology

Haematocrit (HCT)	Lymphocytes (LYMP)
Haemoglobin (HB)	Monocytes (MONO)
Red blood cells (RBC)	Eosinophils (EOS)
Mean cell volume (MCV)	Basophils (BASO)
Mean cell haemoglobin (MCH)	Large unstained cells (LUC)
Mean cell haemoglobin concentration (MCHC)	Erythrocyte sedimentation rate (ESR)
Platelets (PLT)	Reticulocytes (RET)
White blood cells (WBC)	Activated partial thromboplastin time (APTT)
Neutrophils (NEUT)	Prothrombin time (PT)

Table 5.3.1- 23: Biochemistry

Total protein (PROT)	Glucose (GLUC)
Albumin (ALB)	Total cholesterol (CHOL)
Total globulin (GLOB)	Total bilirubin (TBIL)
A/G ratio (A/G)	Chloride (CL)
Calcium (CA)	Aspartate aminotransferase (AST)
Phosphate (PO4)	Alanine aminotransferase (ALT)
Sodium (NA)	Alkaline phosphatase (AP)
Potassium (K)	G-glutamyl transpeptidase (GGT)
Urea (UREA)	Creatine kinase (CPK)
Creatinine (CREA)	

Table 5.3.1- 24: Urinalysis

Appearance (APP)	Ketones (KET)
PH (PH)	Urobilinogen (UBIL)
Protein (PROT)	Bilirubin (BIL)
Glucose (GLUC)	Blood (BLD)
Specific gravity (SG)	Phosphate crystals (PO4)
Bacteria (BACT)	Casts (CAST)
Red blood cells (RBC)	Urate (URAT)
Epithelial cells (EPTH)	White blood cells (WBC)
Sperm (SPER)	Spur deposit colour (SDEP)

5. Sacrifice and pathology

All animals were killed by exsanguination via the carotid artery whilst under deep pentobarbitone anaesthesia. Males were killed on Study Day 30 and females on Study Day 31. One dog was necropsied in turn from each dose group in the following sequence: Group 1, Group 4, Group 2, Group 3. Within each dose group, dogs were necropsied in animal number order.

All dogs were examined thoroughly and macroscopic abnormalities recorded.

6. Organ weights

The following organs from all animals were weighed at necropsy:

Table 5.3.1- 25: Organ weights

Adrenals	Pituitary
Brain	Spleen
Ovaries	Lungs
Kidneys	Heart
Liver	Thyroid
Testes	

The following organs and tissues from all animals were fixed intact or sampled and fixed in 10% neutral buffered formalin, except eyes which were fixed in Davidson's fluid:

Table 5.3.1- 26: Tissue sampling

Adrenals	Liver	Skin + subcutis[3]
Articulated surface and shaft of femur[3]	Lungs	Spinal cord (3 levels)[3]
Aorta[3]	Lymph nodes (mandibular, Mesenteric)[3]	Spleen
Brain	Mammary gland[3]	Sternum
Caecum	Oesophagus	Stomach
Colon	Optic nerve[3]	Testes
Diaphragm[3]	Ovaries + oviducts	Thymus
Duodenum	Pancreas	Thyroid + parathyroid[3]
Epididymides[3]	Pinnae[1]	Tongue[3]
Eyes[3]	Pituitary	Tonsils[3]
Gall bladder[3]	Prostate	Trachea[3]
Heart	Rectum	Urinary bladder
Ileum	Salivary glands (parotid, mandibular + sublingual)	Uterus[2]
Jejunum	Sciatic nerve[3]	Vagina
Kidneys	Skeletal muscle[3]	Any other tissue showing Macroscopic abnormalities
Lacrimal gland[3]		

[1] The ear with tattoo and ID implant was taken for identification purposes only, and was not examined.
[2] Cervix uteri and uterine horns examined
[3] These tissues were collected but not examined microscopically.

A bone marrow smear was taken from all animals and fixed at staining.

5. Histopathology

Following fixation, nominal 5 µm sections of a range of selected organs and tissues from each animal were prepared and stained with haematoxylin and eosin (except bone marrow smear which was stained with Wright's stain). An additional frozen section of the liver from all animals was stained with Oil Red 0 to evaluate the presence of fat.

Tissues were examined for histopathological change with a light microscope by Peter Finn of Precision Histology International, One Eyed Lane, Weybread, Diss, Norfolk, UK. The data were entered directly onto a computer terminal using the Roelce Pathology system which then generated the summary tables and individual animal reports.

II. Results and Discussion

A. Results

1. Clinical results

There were no mortalities and no treatment-related clinical signs.

2. Body weight and food intake

There were no consistent treatment-related effects on body weight or food consumption.

Table 5.3.1- 27: Mean body weights (g)

	Dose level (mg/kg bw/day)			
	0	**10**	**100**	**1,000**
Body weight [g] *(% difference to control)*				
Males				
Day 1	11.2	11.3 *(+0.9)*	11.3 *(+0.9)*	10.9 *(-2.7)*
Day 8	11.6	11.3 *(-2.6)*	11.4 *(-1.7)*	11.2 *(-3.4)*
Day 15	11.8	11.5 *(-2.5)*	11.6 *(-1.9)*	10.9 *(-7.6)*
Day 22	12.2	11.9 *(-2.5)*	11.7 *(-4.1)*	10.9 *(-10.7)*
Day 29	12.2	11.9 *(-2.5)*	11.8 *(-3.3)*	11.1 *(-9.0)*
Females				
Day 1	9.0	9.4 *(+4.4)*	8.5 *(-5.6)*	9.1 *(+1.1)*
Day 8	8.9	9.4 *(+5.6)*	8.4 *(-5.6)*	9.1 *(+2.2)*
Day 15	9.2	9.6 *(+4.3)*	8.5 *(-7.6)*	9.2 *(±0.0)*
Day 22	9.3	9.8 *(+5.4)*	8.5 *(-7.5)*	9.4 *(+1.1)*
Day 29	9.4	9.8 *(+4.3)*	8.5 *(-9.6)*	9.7 *(+3.2)*

Table 5.3.1- 28: Mean food consumption (g/animal/day) *(% difference to control)*

	Dose level (mg/kg bw/day)			
	0	**10**	**100**	**1,000**
Males				
Week 1	400	400 *(±0.0)*	400 *(±0.0)*	400 *(±0.0)*
Week 2	400	400 *(±0.0)*	400 *(±0.0)*	400 *(±0.0)*
Week 3	400	400 *(±0.0)*	400 *(±0.0)*	400 *(±0.0)*
Week 4	400	400 *(±0.0)*	400 *(±0.0)*	400 *(±0.0)*
Females				
Week 1	273	278 *(+1.8)*	284 *(+4.0)*	313 *(+14.7)*
Week 2	322	307 *(-4.7)*	302 *(-6.2)*	318 *(-1.2)*
Week 3	321	325 *(+1.2)*	314 *(-2.2)*	344 *(+7.2)*
Week 4	300	318 *(+6.0)*	305 *(+1.7)*	351 *(+17.0)*

3. Ophthalmoscopic examinations

There were no treatment-related findings.

4. Laboratory investigations

Haematology:

There were no treatment-related findings.

Clinical chemistry:

At 1,000 mg/kg bw/day, there was a slight increase in cholesterol concentration in one male animal (number 947; bold print) on Study Days 14 and 29 when compared with pre-treatment and control values.

There were no other treatment-related effects observed.

Table 5.3.1- 29: Cholesterol measurements (mmol/L)

	Dose level (ppm)															
	Males								Females							
	0		10		100		1,000		0		10		100		1,000	
Animal No.	941	942	943	944	945	946	947	948	949	950	951	952	953	954	955	956
Day -1	4.31	2.22	2.07	3.44	3.65	2.53	3.90	3.17	3.20	3.46	3.37	2.86	3.40	2.43	2.87	3.45
Day 14	3.93	2.38	2.45	3.90	4.02	2.65	**5.56**	3.99	3.22	3.41	3.36	2.68	3.76	2.36	3.55	3.44
Day 29	4.21	2.80	2.50	3.97	4.01	2.62	**7.72**	3.79	3.50	3.34	3.47	2.82	3.80	2.22	3.50	3.35

Urinalysis:

There were no treatment-related findings.

5. Sacrifice, pathology

At 1,000 mg/kg bw/day, an enlarged liver was noted for one male only (number 947).

6. Organ weights

At 1,000 mg/kg bw/day in one male only (number 947), absolute liver weight and liver weight relative to body weight was increased by 34% and 44%, respectively, when compared to the mean values for the controls

7. Histopathology

There were no treatment-related histopathological changes.

III. Conclusion

In the absence of histopathological correlates and based upon the fact that the minor hepatic changes (slight increase in cholesterol and increased absolute and relative liver weight) were observed at 1,000 mg/kg bw/d in a single male only, it is considered that the NOAEL under the conditions of this study was 1,000 mg/kg bw/day.

Assessment and conclusion by applicant:

Study is a dose-range finding study conducted according to the principles of OECD 409 and is acceptable to give some indication of the short-term (28-day) toxicity of fluopicolide in the dog, and to determine the doses for the main study. A NOAEL of 1000 mg/kg bw/d was determined from this study (the highest dose tested).

CA 5.3.2 Oral 90-day study

Data Point:	KCA 5.3.2/01
Report Author:	[redacted]
Report Year:	2000
Report Title:	AE C638206 - Rat 90-day dietary toxicity study with 4 week off-dose period
Report No:	C008603
Document No:	M-197622-01-1
Guideline(s) followed in study:	US-EPA OPPTS 870.3100 (1998); OECD 408 (1998); EEC Commission Directive 88/302/EEC, Annex V, Part B, (1987); JMAFF (1985)
Deviations from current test guideline:	Method: Deviations from current guideline SANCO/3029/99 rev. 4: There is no linearity data presented and the accuracy and precision data are determined from the procedural recoveries in the toxicological studies. However, there are at least 14 recoveries at the LOQ level and at the highest fortification level with mean accuracies between 70-110% and RSD < 20%. Considering that this analytical method is validated in support of toxicological cold studies, the method validation is considered fit for purpose. Study: Deviations from the current OECD guideline (408, 2018): - Blood/plasma samples were not taken for possible analysis of thyroid hormones - At termination, an evaluation of oestrus cycle was not performed. Oestrous cyclicity was evaluated in the rat two-generation study (Blee, M. A. B.; 2003; M-232532-01-1) at dose levels of up to 2,000 ppm (equivalent to at least 100 mg/kg bw/day in F0/F1 females). These deviations do not affect the overall acceptability of the study.
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

Groups of 10 male and 10 female Sprague Dawley rats were fed diet containing 0, 100, 1,400 or 20,000 ppm (equivalent to 0, 7.9, 114 or 1671 mg/kg bw/day for the combined sexes, respectively) of technical fluopicolide for at least 13 weeks. An additional 10 males and 10 females, fed either 0 or 20,000 ppm for 13 weeks, were maintained on control diet for a further 4 weeks to examine the reversibility of any effects seen.

At 20,000 ppm, treatment-related clinical signs included hair loss in both sexes, body soiling and loss of coat condition in males only and a soiled urogenital region in females. Absolute body weight after 13 weeks of treatment was reduced by 30% and 18% compared to the controls in males and females, respectively. Body weight gain over the course of treatment (Day 1-92) was reduced by 41% in males and 29% in females, whilst mean food consumption was reduced by 17% in males and 15% in females and mean food conversion ratios (Week 1-13) were reduced by 28% and 19% compared to control in males and females, respectively. Water intake was increased by 44% during Week 4 of treatment in females only. Haematological investigations indicated slight decreases in haematocrit, haemoglobin, mean cell haemoglobin and mean cell haemoglobin concentration in both sexes, and APTT was slightly increased in males only. There was a slight increase in cholesterol, total protein and GGT in both sexes.

Urinalysis revealed a slight increase in the number of epithelial cells in the urinary sediment of males only and a slight increase in urinary volume and a slight decrease in specific gravity for females only.

Absolute liver weight was increased by 22% in females only and liver weight relative to body weight was increased by 51% in males and 49% in females compared to control. Absolute spleen weight was decreased by 45% and 40% in males and females, respectively, and spleen weight relative to body weight was decreased by 24% in males and 29% in females, when compared to controls. Kidney weight relative to body weight was increased by 15% in males only when compared to controls. Macroscopic examination at necropsy revealed a speckled appearance in both kidneys of 4/10 males. Microscopic examination showed an increase in the severity and incidence of hypertrophy of the zona glomerulosa in the adrenal, trabecular hyperostosis of the bone joint and decreased cellularity of the bone marrows for males and females. Centrilobular hepatocytic hypertrophy, scored as minimal to moderate, was seen in the liver of 9/10 males and 8/10 females. Males only had a number of kidney effects, consisting of an increase in the severity and incidence of accumulation of hyaline droplets in the proximal tubule, single cell death in the proximal tubule epithelium, foci of basophilic (regenerating) tubules and granular casts.

Following the four week off-dose period there was a complete or partial recovery of all treatment-related affects.

At 1,400 ppm, there was a slight increase in cholesterol and a slight increase in the number of epithelial cells in the urinary sediment of males only, and a slight increase in urinary volume and a slight decrease in specific gravity for females only.

Liver weight relative to body weight was increased by 15% in males only, when compared to controls. Absolute spleen weight was decreased by 10% and 16% in males and females respectively, and spleen weight relative to body weight was decreased by 19% in females only, when compared to controls. Kidney weight relative to body weight was increased by 11% in males only when compared to controls.

Macroscopic examination at necropsy revealed a speckled appearance in both kidneys of 3/10 males, with a further 2/10 and 1/10 males having a speckled appearance in the right and left kidneys, respectively. Microscopic examination showed minimal to slight hypertrophy of centrilobular hepatocytes in 8/10 males only and an increase in the severity and incidence of trabecular hyperostosis of the bone joint in females. Kidney effects, in males only, consisted of an increase in severity of accumulation of hyaline droplets in the proximal tubule, single cell death in the proximal tubule epithelium and foci of basophilic (regenerating) tubules and granular casts.

At 100 ppm no effects of treatment were seen.

The NOAEL in the 90-day dietary study in rats was 100 ppm (7.4 or 8.4 mg/kg bw/day in male and female rats respectively) based on treatment-related haematological (reduced haemoglobin and haematocrit in male rats), clinical chemistry (increased cholesterol in male rats at 1,400 ppm), urinalysis (increased urine volume and specific gravity in females at ≥ 1,400 ppm), organ weight (increase in relative liver and kidney weight in male rats and in relative spleen weight in females at 1,400 ppm) and histopathological changes in the liver and kidneys at 1,400 ppm (equivalent 109 or 119 mg/kg bw/day in males and females respectively).

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 technical (fluopicolide)
Purity:	96.9 and 97.5%
Batch no.:	1C990005

2. Vehicle and/or positive control

Vehicle:	Diet

3. Test animals

Species:	Rats
Strain:	Sprague-Dawley Crl: CD IGS BR strain
Age:	28-35 days at receipt 34-41 day at day 1 (start of treatment)
Weight at start:	74-94 g for males, 72-92 g for females at receipt 119-153 g for males, 104-144 g for females at day 1 (start of treatment)
Source:	[redacted]
Acclimation period:	6 days
Diet:	Modified SQC Expanded Ground Rat and Mouse Maintenance Diet No. 1 supplied by Special Diet Services Ltd., Stepfield, Witham, Essex
Water:	Water ad lib
Housing:	Animals were housed in groups of five by sex and dose group under controlled environmental conditions.
Temperature:	19 – 23 °C
Humidity:	45 to 65%
Air changes:	Not given
Photoperiod:	12 hours

B. Study design

1. In-life dates: July 6 to November 10, 1999

2. Animal assignment and treatment

The rats were randomized and assigned to the following test groups.

Table 5.3.2- 1: Study design

Group no.	Dose (ppm)	Number of males	Number of females
Treatment phase			
1	0	10	10
2	100	10	10
3	1,400	10	10
4	20,000	10	10
Recovery phase			
1	0	10	10
4	20,000	10	10

After 13 weeks of continuous dietary exposure, animals from the main groups were killed and those off-dose (recovery) were fed untreated (control) diet for a further 4 weeks and then killed (after a total of 17 weeks on study.

3. Diet preparation and analysis

Prior to the start of treatment, a procedure was developed to prepare homogeneous and suitably stable mixtures of the test material in the laboratory rodent diet at the required nominal concentrations.

Test diets were prepared weekly throughout the study. On each occasion, for each dose level, a pre-mix was made using a grinder and food processor. This in turn was then mixed with remaining laboratory rodent diet and blended in a turbula mixer to produce the required dietary concentration. Subsequent concentrations were prepared by serial dilution.

Every week, aliquots of each concentration (i.e. 100, 1,400 and 20,000 ppm) of the freshly prepared test diets were submitted to Dose Analysis in the Toxicology Function of Aventis CropScience UK Limited (formerly AgrEvo UK Limited) at Chesterford Park for analysis of the test material concentration. Samples were received in powder form in plastic bags and stored deep frozen from time of receipt.

Samples (approx. 200 g) were supplied for analysis from:

i) A trial mix at 100, 1,400 and 20,000 ppm for homogeneity testing and for stability testing after storage at room temperature for 0,1, 8 and 15 days.

ii) The mixes prepared for weeks 1 to 14 of the study.

All the homogeneity samples from the trial mix were analysed. The trial mix stability samples from days 0, 8 and 15 were analysed from the 100 ppm level and the trial mix stability samples from days 0 and 15 were analysed from the 1,400 and 20,000 ppm levels. The week 1, 5, 10 and 14 mixes were analysed at all levels.

The mean results for the test diet samples analysed were within the range 94.3% to 105.9% of nominal (acceptable range +10% to -15% of nominal).

Homogeneity was shown to be satisfactory at all levels, i.e. mean values obtained for top, middle and bottom samples were within the acceptable range 90% to 110% of nominal and these mean % nominal values differed by < 10%.

Stability was satisfactory over the time of use of the diet (8 days), i.e. % nominal levels declined by a maximum of 7% over 15 days storage at room temperature.

4. Statistics

The significance of differences between control and treated groups was analysed by either parametric or non-parametric statistical tests as appropriate. A maximum 2-tailed probability value of 5% ($p < 0.05$) was considered statistically significant.

Body weight, food consumption, water consumption, organ weights, motor activity and assessments from the functional observations battery were analysed for homogeneity of group variances using Bartlett's test. If not significant at the 5 % level, the data were then analysed by one way analysis of variance to establish the significance of variability amongst the groups. If significant, pair-wise comparisons of each treated group in turn with the controls were made using Dunnett's test of significance. Where group variances were heterogeneous using Bartlett's test, pair-wise comparisons were made using a modified t test. For pair-wise comparisons, significance was tested at the 5 and 1% levels ($p <0.05$, $p <0.01$).

Observations from the functional observations battery where there are ranked assessments that are scored into a limited number of categories (e.g. scored 0, 1, 2, 3) were assessed using the Mann-Whitney test (otherwise known as the Wilcoxon rank sum test) for pair wise comparisons. In the functional observation battery where assessments were scored as either present (0) or absent (1) the number of animals in which a condition is present or absent will be recorded for each group at each observation time, in the form of a 2x2 contingency table. Fisher's Exact Test was used to compare each test article treated group with the vehicle control group. Statistical analyses on functional observations were only conducted where positive findings were seen in 3/5 or more animals per group.

Haematology and clinical chemistry data were subjected to the following statistical evaluation:
Bartlett´s test was used to determine homogeneity of variance between groups; if significant at the 5 % level, a log transformation was applied to the data to attempt to remove the heterogeneity.

If homogeneity of variance was demonstrated on either the untransformed or transformed data, parametric tests to detect significant difference between control and test groups at the 5, 1 or 0.1% level comprised:

- One-Way Analysis of Variance to establish the significance of variability among all groups and
- Student's "t" Test based on a pooled variance estimate, for intergroup comparisons, i.e. control vs each test group.

If significant heterogeneity of variance was indicated, even with transformation, then non-parametric analysis was by the Kruskal-Wallis Rank Test to detect any significant group differences at the 5, 1 or 0.1% level.

C. Methods

1. Observations

Animals were observed daily for abnormal behaviour, including neuro-muscular coordination and physical appearance. Observations were made once each morning and, on Mondays to Fridays except public and company holidays, also in the afternoon. More frequent observations were made as necessary. Detailed observations were conducted once weekly prior to weighing.

Records of observations included the nature, time of onset, severity and duration of any abnormal clinical sign/behaviour.

Animals killed in extremis were subjected to necropsy as soon as possible.

2. Body weight, food and water intake

Each animal was weighed upon receipt, at randomisation, at the start of treatment, at weekly intervals thereafter and at necropsy. During Weeks 4, 8 and 11 of treatment, and at the end of the off-dose period (Week 17 of the study), water consumption was measured over a 4-day period.

3. Ophthalmoscopic examination

Ophthalmological examinations were conducted by a consultant veterinarian on all animals prior to the start of treatment and in Week 13, on animals from the control and highest dose groups scheduled for necropsy after 13 weeks of treatment. Both eyes of each animal were examined using an indirect ophthalmoscope after instillation of 0.5% w/v Mydriacyl.

4. Neurotoxicity assessments

During Week 11 of the study, the appearance, behaviour and functional integrity of all animals scheduled for termination after 13 weeks of treatment were assessed using a Functional Observation Battery (FOB).

The FOB comprised a combination of examinations that assessed the reaction of animals to handling on removal from the cage and proceeded to observations in an open field standard arena. In addition to the FOB, the grip strength of each animal was assessed during Week 12.

The cages were identified by animal number only and the observers were unaware of the experimental group to which the animal belonged. Any deviations from normal were recorded with respect to nature, and where appropriate, incidence or severity.

Functional Observations Battery (FOB):

A clear polycarbonate arena was used for examination in the open field, following parameters were assessed:

Table 5.3.2- 2: FOB parameters

Ease of removal from cage (EREM)	Clonic convulsions (CLON)
Ease of handling (HAND)	Tonic convulsions (TONN)
Body temperature (TEMP)	Tonic involuntary leaping (TONL)
Exophthalmus (EXOS)	Rearing (REAR)
Palpebral closure (PALP)	Defaecation frequency (DEFC)
Pupil response - right eye (PUPR)	Urination frequency (URIN)
Pupil response - left eye (PUPL)	Alertness (ALER)
Lachrymation (LACH)	Approach response (APPR)
Salivation (SALN)	Righting reflex (RRFX)
Vocalisations (VOCS)	Right/left tilt (TILT)
Mobility (MOBI)	Startle response (STRT)
Posture (sways/lurches) (SWAY)	Landing foot splay (LFSM)
Hind limbs splayed (SPLY)	Grip strength - fore limb (GRPF)
Hind limbs dragging (DRAG)	Grip strength - hind limb (GRPH)
Hunched posture (HNCH)	Tail pinch (TPCH)
Ataxia (ATAX)	

General observations - included any other relevant information, e.g. dehydration, emaciation etc.

Motor activity:

The locomotor behaviour of all animals scheduled for termination after 13 weeks of treatment was measured during week 12. Activity was monitored using Ethovision™ (Video tracking, Motion analysis and behaviour recognition system). The experimental apparatus consisted of one video camera (Panasonic WV-CP412E) suspended from the ceiling above a group of cages, which were held within a black wooden template. This was connected to a separate video recorder (Panasonic AG-5700b) which in turn was attached to a single colour monitor (Panasonic CWCM1450).

In the first session the first two animals numerically from each group were monitored. In each consecutive period the next animals from each group were monitored in numerical order. The video recorder was activated to record animals over a 60 minute period. This procedure was repeated for each session. The analogue video images from each tape were translated into digital information using the Ethovision™ software, and then analysed to derive the total distance moved (cm) by each rat during the whole 60 minute period.

5. Laboratory investigations (haematology, clinical chemistry, urinalysis)

Samples for haematology and clinical chemistry analyses were collected during week 13 and at the end of the off-dose period (week 17 of the study) from the retro-orbital sinus of each animal whilst under isoflurane anaesthesia.

Urine samples were obtained from each animal overnight during Weeks 12 and 17.

Animals were housed in individual urine collecting cages and all the urine voided overnight was collected. Animals were deprived of food during the collection period.

The parameters listed below were measured or derived:

Table 5.3.2- 3: Haematology

Haematocrit (HCT)	White blood cells (WBC)
Haemoglobin (HB)	Neutrophils (NEUT)
Red blood cells (RBC)	Lymphocytes (LYMP)
Mean cell volume (MCV)	Monocytes (MONO)
Mean cell haemoglobin (MCH)	Eosinophils (EOS)
Mean cell haemoglobin concentration (MCHC)	Basophils (BASO)
	Large unstained cells (LUC)
Platelets (PLT)	Reticulocyte count (RET)
Pro-thrombin time (PT)	Activated partial thromboplastin time (APTT)

Table 5.3.2- 4: Biochemistry

Total protein (PROT)	Total cholesterol (CHOL)
Albumin (ALB)	Total bilirubin (TBIL)
Total globulin (GLOB)	Chloride (CL)
A/G ratio (A/G)	Aspartate aminotransferase (AST)
Calcium (CA)	Alanine aminotransferase (ALT)
Phosphate (PO4)	Alkaline phosphatase (AP)
Sodium (NA)	G-glutamyl transpeptidase (GGT)
Potassium (K)	Creatine kinase (CPK)
Urea (UREA)	
Creatinine (CREA)	
Glucose (GLUC)	

Table 5.3.2- 5: Urinalysis

PH (PH)	Bacteria (BACT)
Protein (PROT)	Red blood cells (RBC)
Glucose (GLUC)	Epithelial cells (EPTH)
Ketones (KET)	Phosphate crystals (P04)
Urobilinogen (UBIL)	Urate crystals (URAT)
Bilirubin (BIL)	Casts (CAST)
Blood (BLD)	White blood cells (WBC)
Appearance (APP)	Sperm (SPER)
Volume (VOL)	Spun deposit colour (SDEP)
Specific gravity (SG	

6. Sacrifice and pathology

Animals were killed by exsanguination via the abdominal aorta under ether anaesthesia. All animals were examined thoroughly and macroscopic abnormalities recorded.

Organ weights:

The following organs from all animals were weighed at necropsy:

Table 5.3.2- 6: Organ weights

Liver	Brain
Kidneys	Adrenals
Spleen	Heart
Testes	Thymus
Ovaries	Uterus
Epididymides	

Tissue sampling:

The following organs and tissues from all animals were fixed in 10% neutral buffered formalin except eyes which were fixed in Davidson's fluid:

Table 5.3.2- 7: Tissue sampling and processing

Adrenals	Liver	Sciatic nerve
Aorta	Lungs	Skeletal muscle
Brain	Lymph nodes (cervical &	Skin + subcutis
Caecum	Mesenteric)	Spinal cord (3 levels)
Coagulating gland	Mammary gland (females)	Spleen
Colon	Nose/turbinates	Sternum
Diaphragm	Oesophagus	Stomach
Duodenum	Optic nerve	Seminal vesicle
Epididymides	Ovaries	Testes
Eyes	Oviducts	Thymus
Femur joint	Pancreas	Thyroid + parathyroid
Harderian gland	Pharynx	Tongue
Head (fixed only)	Pinnae[1]	Trachea
Heart	Pituitary	Urinary bladder
Ileum	Prostate	Uterus[2]
Jejunum	Rectum	Vagina
Kidneys	Salivary glands (parotid,	Any other tissue showing
Lacrimal gland	sublingual + submaxillary)	macroscopic abnormalities
Larynx		

[1] Taken for identification purposes only; not examined
[2] Uterine horns and cervix uteri examined

A bone marrow smear was taken from all animals and fixed subsequently at staining.

7. Histopathology

Tissue processing:

Following fixation, nominal 5 µm sections of all organs and tissues from all main study animals in the control and high dose groups were prepared and stained with haematoxylin and eosin. Cryostat liver sections were stained with Oil Red O to identify and quantify the presence of any fat deposits.

In addition, the liver and liver Oil Red O sections, kidneys, lungs, adrenals, bone joint and bone marrow from the femur and sternum, respectively, were examined from all animals in the low and intermediate dose groups. The caecum of females in all the dose groups was also examined. Any macroscopic abnormalities were examined from all dose groups except off-dose animals.

Microscopic examination:

Tissue sections were examined for histopathological changes by using a light microscope. Findings were entered directly onto a computerised histopathology database, which subsequently generated summary tables.

Sections from the caecum, bone (stifle joint, femur and tibia), and sternal bone marrow were peer reviewed by J.P. Finn of Precision Histology International, London Road, Harleston, Norfolk, UK.

II. Results and Discussion

A. Results

1. Clinical results

At 20,000 ppm, treatment-related clinical signs included hair loss in both sexes, body soiling and loss of coat condition in males only, and a soiled urogenital region in females. There were no treatment-related clinical signs at the lower dose levels.

Treatment-related clinical signs that persisted during the off-dose period were hair loss in both sexes and urogenital soiling in females. Two mortalities in this study were incidental and unrelated to treatment.

2. Body weight and food and water intake

Body weight

At 20,000 ppm, absolute body weight after 13 weeks of treatment (Day 92) was reduced by 30% and 18% compared to the controls in males and females, respectively. Body weight gain over the course of treatment (Day 1-92) was reduced by 47% in males and 29% in females, when compared to the controls. This effect was most severe during week 1 of treatment (Day 1-8: -98% in males, -95% in females).

There were no relevant effects on body weight or body weight gain at the lower dose levels.

At the end of the recovery period (Day 120), body weight gain of both sexes was considerably greater than the controls (+111% for males, +300% for females).

Table 5.3.2- 8: Mean body weight and body weight gain (g)

	Dose (ppm)			
	0	**100**	**1,400**	**20,000**
MAIN PHASE				
Males				
Body weight [g] *(% difference to control)*				
Day 1	134	137 *(+2.2)*	136 *(+1.5)*	135 *(+0.7)*
Day 8	192	194 *(+1.0)*	183 *(-4.7)*	136** *(-29.2)*
Day 15	252	254 *(+0.8)*	238 *(-5.6)*	175** *(-37.7)*
Day 22	307	308 *(+0.3)*	293 *(+4.6)*	212** *(-30.9)*
Day 29	346	346 *(±0.0)*	323* *(-6.6)*	235** *(-32.1)*
Day 36	385	381 *(+1.0)*	365 *(-5.2)*	268** *(-30.4)*
Day 43	418	414 *(-1.0)*	396 *(-5.3)*	290** *(-30.6)*
Day 50	443	436 *(-1.6)*	419 *(-5.4)*	306** *(-30.9)*
Day 57	461	463 *(+0.4)*	443 *(-3.9)*	325** *(-29.5)*
Day 64	483	480 *(-0.6)*	458 *(-5.2)*	334** *(-30.8)*
Day 71	501	500 *(-0.2)*	475 *(-5.2)*	342** *(-31.7)*
Day 78	520	520 *(±0.0)*	492 *(-5.4)*	358** *(-31.2)*
Day 85	528	523 *(-0.9)*	495 *(-6.3)*	366** *(-30.7)*
Day 92	543	533 *(-1.8)*	512 *(-5.7)*	378** *(-30.4)*
Body weight gain [g] *(% difference to control)* +				
Day 1-8	58	57 *(-1.7)*	47 *(-19.0)*	1 *(-98.3)*
Day 1-15	118	117 *(-0.8)*	102 *(-13.6)*	40 *(-66.1)*
Day 1-22	173	171 *(-1.2)*	157 *(-9.2)*	77 *(-55.5)*
Day 1-92	409	396 *(-3.2)*	376 *(-8.1)*	243 *(-40.6)*
Females				
Body weight [g] *(% difference to control)*				
Day 1	125	125 *(±0.0)*	124 *(-0.8)*	123 *(-1.6)*
Day 8	164	162 *(-1.2)*	160 *(-2.4)*	125** *(-23.8)*
Day 15	196	189 *(-3.5)*	187 *(-1.6)*	153## *(-19.5)*
Day 22	211	212 *(+0.5)*	211 *(±0.0)*	176** *(-16.6)*
Day 29	229	230 *(+0.4)*	228 *(-0.4)*	193** *(-15.7)*
Day 36	248	247 *(-0.4)*	248 *(±0.0)*	208** *(-16.1)*
Day 43	261	259 *(-0.8)*	260 *(-0.4)*	220** *(-15.7)*
Day 50	270	270 *(±0.0)*	267 *(-1.1)*	227** *(-15.9)*
Day 57	282	282 *(±0.0)*	280 *(-0.7)*	235** *(-16.7)*
Day 64	287	287 *(±0.0)*	283 *(-1.4)*	237** *(-17.4)*
Day 71	293	292 *(-0.3)*	288 *(-1.7)*	240## *(-18.1)*
Day 78	299	300 *(+0.3)*	298 *(-0.3)*	246## *(-17.7)*
Day 85	302	300 *(-0.7)*	297 *(-1.7)*	247## *(-18.2)*
Day 92	306	304 *(-0.7)*	300 *(-2.0)*	251## *(-18.0)*

	Dose (ppm)			
	0	**100**	**1,400**	**20,000**
Body weight gain [g] *(% difference to control)* +				
Day 1-8	39	37 *(-5.1)*	36 *(-7.7)*	2 *(-94.9)*
Day 1-15	65	64 *(-1.5)*	63 *(-3.1)*	30 *(-53.8)*
Day 1-22	86	87 *(+1.2)*	87 *(+1.2)*	53 *(-38.4)*
Day 1-92	181	179 *(-1.1)*	176 *(-2.8)*	128 *(-29.3)*
RECOVERY PHASE				
Males				
Body weight [g] *(% difference to control)*				
Day 99	564	-	-	406** *(-28.0)*
Day 106	579	-	-	423** *(-26.9)*
Day 113	582	-	-	436** *(-25.1)*
Day 120	583	-	-	446** *(-23.5)*
Body weight gain [g] *(% difference to control)* +				
Day 99-120	19	-	-	40 *(+110*)*
Females				
Body weight [g] *(% difference to control)*				
Day 99	321	-	-	258** *(-19.6)*
Day 106	326	-	-	263** *(-19.3)*
Day 113	326	-	-	265** *(-18.7)*
Day 120	323	-	-	266** *(-17.6)*
Body weight gain [g] *(% difference to control)*				
Day 99-120	2	-	-	8 *(+300)*

* / ** p < 0.05 / p < 0.01, statistically different to control using Dunnet's test
# / ## p < 0.05 / p< 0.01, statistically different to control using modified t-test
+ No statistical analyses were performed for body weight gains
- : Dose levels not analysed during recovery phase

Food and water intake

At the end of the treatment period (Week 13), mean food consumption of the 20,000 ppm dose group animals was reduced by 17% in males and 15% in females, when compared to controls. This effect was more severe during the first week of treatment, when food consumption was reduced by 54% and 48% compared to controls for males and females, respectively.

At the lower dose levels, no effects on food consumption were observed.

At the end of the recovery period (Week 17), the mean food intake of males of the 20,000 ppm dose group was slightly reduced (-4% compared to control) whilst the mean food intake of females was similar to that of the control animals.

Table 5.3.2- 9: Mean food consumption (g/animal/day) *(% difference to control)*

	Dose level (ppm)			
	0	**100**	**1,400**	**20,000**
MAIN PHASE				
Males				
Week 1	26	26 *(±0.0)*	24* *(-7.7)*	12** *(-53.8)*
Week 4	30	29 *(-3.3)*	29 *(-3.3)*	24** *(-20.0)*
Week 8	29	28 *(-3.4)*	28 *(-3.4)*	24** *(-17.2)*
Week 13	29	27 *(-6.9)*	28 *(-3.4)*	24** *(-17.2)*
Females				
Week 1	21	21 *(±0.0)*	21 *(±0.0)*	11** *(-47.6)*
Week 4	21	21 *(±0.0)*	21 *(±0.0)*	18** *(-14.3)*
Week 8	21	21 *(±0.0)*	21 *(±0.0)*	18** *(-15.0)*
Week 13	20	21 *(+5.0)*	20 *(±0.0)*	17** *(-15.0)*
RECOVERY PHASE				
Males				
Week 17	24	-	-	23 *(-4.2)*
Females				
Week 17	17	-	-	17 *(±0.0)*

* p < 0.05 , ** p < 0.01, statistically different to control using Dunnett's test
- : Dose levels not analysed during recovery phase

At 20,000 ppm, mean food conversion ratios over the course of the study (mean values Week 1-13) were reduced by 28% and 19% compared to controls in males and females, respectively.

Table 5.3.2- 10: Mean food conversion (%)

	Dose level (ppm)			
	0	**100**	**1,400**	**20,000**
Males				
Week 1	32.0	31.4	29.1	0.6
Week 2	29.5	29.8	27.9	26.1
Week 3	25.9	26.1	26.7	22.1
Week 4	18.8	18.3	14.8	13.2
Week 5	17.8	17.2	19.6	16.9
Week 6	16.4	17.0	16.0	13.4
Week 7	12.4	11.6	11.5	9.8
Week 8	8.6	13.6	12.4	11.2
Week 9	10.9	9.0	7.8	5.6
Week 10	8.7	10.4	8.4	5.2
Week 11	9.8	10.2	9.1	10.0
Week 12	3.7	2.0	1.3	5.0
Week 13	7.5	4.9	9.0	6.7
Mean (Week 1-13) *(% difference to control)*	**15.54**	**15.50** *(-0.3)*	**14.89** *(-4.2)*	**11.22** *(-27.8)*

	Dose level (ppm)			
	0	100	1,400	20,000
Females				
Week 1	26.3	25.4	24.4	2.6
Week 2	18.1	18.6	18.5	21.6
Week 3	15.4	16.3	16.5	17.4
Week 4	12.4	11.9	11.4	12.9
Week 5	12.2	10.8	12.7	10.9
Week 6	9.1	8.2	7.8	9.6
Week 7	6.2	7.0	4.9	5.2
Week 8	7.7	8.9	9.3	6.9
Week 9	3.7	3.5	2.1	1.3
Week 10	4.6	4.1	3.9	3.3
Week 11	4.4	5.4	6.5	5.1
Week 12	2.2	0.3	-0.7	0.6
Week 13	2.4	2.7	2.1	3.0
Mean (Week 1-13) *(% difference to control)*	**9.59**	**9.47** *(-1.2)*	**9.18** *(-4.3)*	**7.74** *(-19.3)*

At 20,000 ppm, water intake of females was increased by 44% compared to control during Week 4 of treatment only. At lower doses (1,400 and 100 ppm), water consumption of females remained unaffected. In males, no effects on water consumption were observed at any dose level.

Table 5.3.2- 11: Mean water consumption (g/animal/day) *(% difference to control)*

	Dose level (ppm)			
	0	100	1,400	20,000
Males				
Week 4	29	29 *(±0.0)*	32 *(+10.3)*	30 *(+3.4)*
Week 8	32	31 *(-3.1)*	39 *(+21.9)*	31 *(-3.1)*
Week 11	31	32 *(+3.2)*	38 *(+22.6)*	31 *(±0.0)*
Females				
Week 4	23	27 *(+17.4)*	28 *(+21.7)*	33** *(+43.5)*
Week 8	30	30 *(±0.0)*	32 *(+6.7)*	36 *(+20.0)*
Week 11	27	28 *(+3.7)*	30 *(+11.1)*	31 *(+14.8)*

* p < 0.05 ** p < 0.01; statistically different to control using Dunnett's test

3. Ophthalmoscopic examinations

There were no treatment-related ophthalmic lesions.

4. Neurotoxicity assessments

Functional Observations Battery:

There were no treatment-related effects on the functional integrity or behaviour of the animals.

Grip Strength:

There were no treatment-related findings.

Motor activity:

There were no treatment-related findings.

5. Laboratory investigations

Haematology:

At 20,000 ppm, haemoglobin concentration, haematocrit, mean cell haemoglobin and mean cell haemoglobin concentration were slightly decreased in both sexes whilst activated partial thromboplastin time was slightly increased in males only when compared with controls.

Other statistically significant differences were considered to be of no toxicological significance because they were either not dose-related, or the values were within the normal range of historical controls.

After 4 weeks off-dose, the previously affected haematological values in both sexes were mostly comparable to controls.

Table 5.3.2- 12: Haematological results in males and females at termination and at the end of the recovery period *(% difference to control)*

	Dose level (ppm)					
	0	**100**		**1,400**		**20,000**	
Males							
Week 13							
Haemoglobin (g/L)	152	154	*(+1.3)*	145*	*(-4.6)*	143*	*(-5.9)*
Haematocrit (L/L)	0.45	0.45	*(+0.0)*	0.43*	*(-4.4)*	0.43*	*(-4.4)*
MCH (pg)	17.9	18.3	*(+2.2)*	17.5	*(-2.2)*	17.0**	*(-5.0)*
MCHC (G/L)	342	341	*(-0.3)*	338	*(-1.2)*	332**	*(-2.9)*
Recovery - week 17							
Haemoglobin (g/L)	152	-	-	-	-	147	*(-3.3)*
Haematocrit (L/L)	0.44	-	-	-	-	0.43	*(-2.3)*
MCH (pg)	17.6	-	-	-	-	17.4	*(-1.3)*
MCHC (G/L)	348	-	-	-	-	344*	*(-1.1)*

	Dose level (ppm)					
	0	100		1,400		20,000	
Females						
Week 13						
Haemoglobin (g/L)	151	152	*(+0.7)*	148	*(-2.0)*	137***	*(-9.3)*
Haematocrit (L/L)	0.43	0.44	*(+2.3)*	0.43	*(±0.0)*	0.41**	*(-4.7)*
MCH (pg)	18.8	18.7	*(-0.5)*	18.9	*(+0.5)*	17.1***	*(-9.0)*
MCHC (G/L)	349	350	*(+0.3)*	346	*(-0.9)*	340**	*(-2.6)*
Recovery - week 17							
Haemoglobin (g/L)	149	-	-	-	-	145	*(-3.4)*
Haematocrit (L/L)	0.42	-	-	-	-	0.42	*(±0.0)*
MCH (pg)	18.8	-	-	-	-	18.3*	*(-2.7)*
MCHC (G/L)	354	-	-	-	-	349*	*(-1.4)*

* p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, statistically different to control
- : Dose levels not analysed during recovery phase

Clinical chemistry:

At 20,000 ppm, there was a slight increase in cholesterol concentration, total protein and gamma-glutamyl transferase activity in males and females.

At 1,400 ppm, there was a slight increase in cholesterol concentration for males only.

Other statistically significant differences were considered to be of no toxicological significance because they were either not dose-related, or the values were within the normal range of historical controls.

After 4 weeks off-dose, all previously affected clinical chemistry values in both sexes were comparable to controls.

Table 5.3.2- 13: Selected biochemistry results at the end of the study and recovery period *(% difference to control)*

Week	Dose level (ppm)			
	0	100	1,400	20,000
Males				
Week 13				
Total protein (g/L)	64.3	65.7 *(+2.2)*	66.0 *(+2.6)*	70.5** *(+9.6)*
Cholesterol (mmol/L)	1.63	1.94* *(+19.0)*	2.23*** *(+36.8)*	3.63*** *(+122.7)*
Recovery - Week 17				
Total protein (g/L)	63.8	-	-	62.6 *(-1.9)*
Cholesterol (mmol/L)	1.75	-	-	1.46* *(-16.6)*
Females				
Week 13				
Total protein (g/L)	68.4	67.0 *(-2.0)*	69.5 *(+1.6)*	73.5** *(+7.5)*
Cholesterol (mmol/L)	2.11	2.11 *(±0.0)*	2.26 *(+7.1)*	3.51*** *(+66.4)*
Recovery - Week 17				
Total protein (g/L)	64.6	-	-	66.6 *(+3.1)*
Cholesterol (mmol/L)	1.89	-	-	2.14 *(+13.2)*

* p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, statistically different to control
- : Dose levels not analysed during recovery phase

Urinalysis:

At 20,000 ppm, there was a slight increase in the number of epithelial cells in the urinary sediment of males only. There was a slight increase in urinary volume and a slight decrease in specific gravity in females only.

At 1,400 ppm there was a slight increase in the number of epithelial cells in the urinary sediment of males only. There was a slight increase in urinary volume and a slight decrease in specific gravity in females only.

There were no treatment-related effects at 100 ppm in either sex.

After 4 weeks off-dose, the urinary volume of females was still slightly increased when compared with conrols. All other previously affected urinalysis parameters were comparable to controls.

4. Sacrifice and pathology

Necropsy:

At 20,000 ppm, 4/10 males killed after 13 weeks had a speckled appearance in both kidneys. At 1,400 ppm, 3/10 males killed after 13 weeks had a speckled appearance in both kidneys, with a further 2/10 and 1/10 males having a speckled appearance in the right and left kidneys respectively.

At 100 ppm, speckled kidney was observed in one male animal (right). Due to its isolated occurance and in the absence of any correlating histopathological findings this finding is considered incidental and not adverse. Therefore, no treatment-related macroscopic abnormalities were observed at this dose level.

In females, no necropsy kidney findings were observed at any dose level.

After 4 weeks off-dose 3/10 males previously given 20,000 ppm were found to have a speckled appearance in both kidneys, with a further 1/10 males having a speckled appearance in the right kidney.

Table 5.3.2- 14: Necropsy findings - kidney (13 weeks) – Number of animals affected

Finding	Dose level (ppm)			
	0	100	1,400	20,000
Males				
Speckled kidney - left	0	0	4	4
Speckled kidney - right	0	1	5	4
Total	**0**	**1**	**9**	**8**
Females				
Speckled kidney - left	0	0	0	0
Speckled kidney - right	0	0	0	0
Total	**0**	**0**	**0**	**0**

Organ weights:

At 20,000 ppm, absolute liver weight was increased by 22% compared to control in females only whilst relative liver was increased by 51% in males and 49% in females compared to control. Absolute spleen weight was decreased by 45% and 40% in males and females, respectively, and relative spleen weight, relative was decreased by 24% in males and 29% in females compared to control.

In addition, several absolute organs weights were statistically significantly decreased while relative weights were increased or not changed (adrenals, kidney, epididymis, testes, ovaries). Therefore, these organ weight changes are considered as an indirect effect of the markedly reduced bodyweight at the hightest dose level.

At 1,400 ppm, liver weight relative to body weight was increased by 13% in males only, when compared to controls. Absolute spleen weight was decreased by 10% and 16% in males and females, respectively whilst for females only, spleen weight relative to body weight was decreased by 19% compared to control. Relative kidney weight was increased by 11% in males only.

At 100 ppm, no effects on organ weight were noted in either sex.

After 4 weeks off-dose, there was a trend for a reversal of the effects on the liver and spleen weights of males and females and the kidney weights of males.

Table 5.3.2- 15: Mean absolute and relative organ weights *(% difference to control)*

		Dose level (ppm)					
		0	100		1,400	20,000	
		Males					
Week 13							
Terminal body weight (g)		527.0	526.5	*(-0.1)*	501.6	355.5	*(-32.5)*
Adrenals	abs. (g)	0.061	0.062	*(-1.6)*	0.053	0.042**$	*(-31.1)*
	rel. (%)	0.012	0.012	*(±0.0)*	0.011	0.012	*(±0.0)*
Liver	abs. (g)	16.44	15.99	*(-2.7)*	17.99	16.75	*(+1.9)*
	rel. (%)	3.13	3.02	*(-3.5)*	3.59**	4.71**	*(+50.5)*
Kidney	abs. (g)	2.82	2.77	*(-1.8)*	3.02	2.20**$	*(-22.0)*
	rel. (%)	0.54	0.53	*(-1.9)*	0.60*	0.62**$	*(+14.8)*
Spleen	abs. (g)	0.87	0.88	*(+1.1)*	0.78	0.48**	*(-44.8)*
	rel. (%)	0.17	0.17	*(±0.0)*	0.16	0.13*	*(-23.5)*
Testes	abs. (g)	3.73	3.66	*(-1.9)*	3.34*	3.47	*(-7.0)*
	rel. (%)	0.71	0.70	*(-1.4)*	0.67	0.98**$	*(+38.0)*
Epididymis	abs. (g)	1.285	1.257	*(-2.2)*	1.218	1.142*$	*(-11.1)*
	rel. (%)	0.245	0.241	*(-1.6)*	0.245	0.322**$	*(+31.4)*

		Dose level (ppm)							
		0	100		1,400		20,000		
Recovery – Week 17									
Terminal body weight (g)		575.9	-	-	-	-	442.2	*(-23.2)*	
Adrenals	abs. (g)	0.059	-	-	-	-	0.045**$	*(-23.7)*	
	rel. (%)	0.010	-	-	-	-	0.010	*(±0.0)*	
Liver	abs. (g)	16.98	-	-	-	-	13.98**$	*(-17.7)*	
	rel. (%)	2.95	-	-	-	-	3.15#	*(+6.8)*	
Kidney	abs. (g)	2.99	-	-	-	-	2.45**$	*(-18.1)*	
	rel. (%)	0.52	-	-	-	-	0.55	*(+5.8)*	
Spleen	abs. (g)	0.88	-	-	-	-	0.67**$	*(-23.9)*	
	rel. (%)	0.15	-	-	-	-	0.15	*(±0.0)*	
Testes	abs. (g)	3.47	-	-	-	-	3.56	*(+2.6)*	
	rel. (%)	0.61	-	-	-	-	0.81**$	*(+32.8)*	
Epididymis	abs. (g)	1.366	-	-	-	-	1.225	*(-10.3)*	
	rel. (%)	0.239	-	-	-	-	0.280**$	*(+17.2)*	
Females									
Week 13									
Terminal body weight (g)		301.1	301.9	*(±0.0)*	297.3	*(-1.3)*	247.7	*(-17.7)*	
Adrenals	abs. (g)	0.072	0.074	*(+2.8)*	0.080	*(+11.1)*	0.060*$	*(-16.7)*	
	rel. (%)	0.024	0.024	*(±0.0)*	0.027	*(+12.5)*	0.024	*(±0.0)*	
Liver	abs. (g)	9.68	9.54	*(-1.4)*	10.03	*(+3.6)*	11.79**	*(+21.8)*	
	rel. (%)	3.21	3.16	*(-1.6)*	3.37	*(+5.0)*	4.77**	*(+48.6)*	
Kidney	abs. (g)	1.84	1.76	*(-4.3)*	1.86	*(+1.1)*	1.52**$	*(-17.4)*	
	rel. (%)	0.62	0.58	*(-6.5)*	0.63	*(+1.6)*	0.61	*(-1.6)*	
Spleen	abs. (g)	0.62	0.61	*(-1.6)*	0.52	*(-16.1)*	0.37##	*(-40.3)*	
	rel. (%)	0.21	0.20	*(-4.8)*	0.17**	*(-19.0)*	0.15**	*(-28.6)*	
Ovaries	abs. (g)	0.125	0.150*	*(+20.0)*	0.130	*(+4.0)*	0.101*$	*(-19.2)*	
	rel. (%)	0.042	0.050	*(+19.0)*	0.044	*(+4.8)*	0.041	*(-2.4)*	
Uterus	abs. (g)	0.79	0.63	*(-17.7)*	0.90	*(+13.9)*	0.53	*(-32.9)*	
	rel. (%)	0.27	0.21	*(-22.2)*	0.31	*(+14.8)*	0.21	*(-22.2)*	

		Dose level (ppm)				
		0	100	1,400	20,000	
Recovery – Week 17						
Terminal body weight (g)		320.7	-	-	266.3	*(-17.0)*
Adrenals	abs. (g)	0.079	-	-	0.058**$	*(-26.6)*
	rel. (%)	0.025	-	-	0.022	*(-12.0)*
Liver	abs. (g)	10.04	-	-	9.26	*(-7.8)*
	rel. (%)	3.13	-	-	3.48**$	*(+11.2)*
Kidney	abs. (g)	1.90	-	-	1.65**$	*(-13.2)*
	rel. (%)	0.60	-	-	0.62	*(+3.3)*
Spleen	abs. (g)	0.61	-	-	0.51*$	*(-16.4)*
	rel. (%)	0.19	-	-	0.19	*(±0.0)*
Ovaries	abs. (g)	0.142	-	-	0.128	*(-9.9)*
	rel. (%)	0.045	-	-	0.048	*(+6.7)*
Uterus	abs. (g)	0.63	-	-	0.66	*(+4.8)*
	rel. (%)	0.20	-	-	0.25	*(+25.0)*

* / **: $p \leq 0.05$ / $p \leq 0.01$, statistically different from control (Dunnett's test)
# / ##: $p \leq 0.05$ / $p \leq 0.01$, statistically different from control (modified T-test)
$: considered secondary to decreased bodyweight
abs.: absolute organ weight
rel.: relative organ weight (body weight ratio %)
- : Dose levels not analysed during recovery phase

6. Histopathology

Histopathological changes were seen in the adrenals, bone joint and bone marrow, liver and kidneys.

Adrenals:

At 20,000 ppm, there was an increase in the severity and incidence of hypertrophy of the zona glomerulosa of the adrenal cortex. There were no treatment-related histopathological changes in either sex at 100 or 1,400 ppm.

Table 5.3.2- 16: Adrenal findings – Number of animals affected

Finding / Severity	Dose level (ppm)							
	Males				Females			
	0	100	1,400	20,000	0	100	1,400	20,000
Hypertrophy of the zona glomerulosa								
After 13 weeks:								
- minimal	1	0	0	0	1	1	3	3
- slight	0	0	0	7	0	0	0	7
Total	**1/10**	**0/10**	**0/10**	**7/10**	**1/10**	**1/10**	**3/10**	**10/10**
After off-dose period								
- minimal	0	-	-	8	1	-	-	4
- slight	0	-	-	0	0	-	-	1
Total	**0/10**	**-**	**-**	**8/10**	**1/10**	**-**	**-**	**5/10**

No statistical analyses were performed on histopathological findings.
- : Dose levels not analysed during recovery phase (off-dose period)

Following 4 weeks off-dose the severity of hypertrophy had decreased in both sexes.

Liver:

At 20,000 ppm, minimal to moderate hypertrophy of centrilobular hepatocytes was seen in 9/10 males and 8/10 females.

At 1,400 ppm minimal to slight hypertrophy of centrilobular hepatocytes was present in 8/10 males only.

There were no treatment-related histopathological changes in females at 1,400 ppm, or in either sex at 100 ppm.

Table 5.3.2- 17: Liver findings – Number of animals affected

Finding / Severity	Dose level (ppm)							
	Males				Females			
	0	100	1,400	20,000	0	100	1,400	20,000
Centrilobular hepatocytic hypertrophy								
After 13 weeks:								
- minimal	0	0	3	1	0	0	0	4
- slight	0	0	5	0	0	0	0	4
- moderate	0	0	0	8	0	0	0	0
Total	**0/10**	**0/10**	**8/10**	**9/10**	**0/10**	**0/10**	**0/10**	**8/10**
After off-dose period								
- Not present	10	-		10	10	-	-	10

No statistical analyses were performed on histopathological findings.
- : Dose levels not analysed during recovery phase (off-dose period)

Following 4 weeks off-dose, a complete reversibility of all liver effects was seen.

Bone joint and bone marrow:

At 20,000 ppm, an increase in the severity and incidence of trabecular hyperostosis in the bone joint and a decreased cellularity in the bone marrow for 7/10 males and 9/10 females was recorded.

At 1,400 ppm, there was an increase in the severity and incidence of trabecular hyperostosis in the bone joint for females only.

There were no treatment-related histopathological changes in males at 1,400 ppm, or in either sex at 100 ppm.

Table 5.3.2- 18: Bone joint and bone marrow findings – Number of animals affected

Finding / Severity	Dose level (ppm)							
	Males				Females			
	0	100	1,400	20,000	0	100	1,400	20,000
Trabecular hyperostosis in the bone								
After 13 weeks:								
- minimal	0	0	0	2	3	1	2	1
- slight	0	0	0	3	0	0	3	4
- moderate	0	0	0	2	0	0	3	4
Total	**0/10**	**0/10**	**0/10**	**7/10**	**3/10**	**1/10**	**8/10**	**9/10**
After off-dose period								
- minimal	0	-	-	0	0	-	-	3
- slight	0	-	-	0	1	-	-	3
- moderate	0	-	-	0	0	-	-	1
Total	**0/10**	**-**	**-**	**0/10**	**1/10**	**-**	**-**	**7/10**
Decreased cellularity in the bone marrow								
After 13 weeks:								
- minimal	0	1	0	3	2	0	0	4
- slight	0	0	0	4	0	0	0	5
Total	**0/10**	**1/10**	**0/10**	**7/10**	**3/10**	**0/10**	**0/10**	**9/10**
After off-dose period								
- minimal	0	-	-	3	4	-	-	2
- slight	0	-	-	0	0	-	-	3
Total	**0/10**	**-**	**-**	**3/10**	**4/10**	**-**	**-**	**5/10**

No statistical analyses were performed on histopathological findings.
- : Dose levels not analysed during recovery phase (off-dose period)

Following 4 weeks off-dose, there was a full or partial recovery of the effects seen during the treatment period.

Kidneys:

At 20,000 ppm, there were a number of kidney effects in the males consisting of an increase in incidence and severity of accumulation of hyaline droplets, single cell death in the proximal tubule epithelium and foci of basophilic (regenerating) tubules and granular casts.

At 1,400 ppm, in males only, there was an increase in incidence and severity accumulation of hyaline droplets, single cell death in the proximal tubule epithelium and foci of basophilic (regenerating) tubules and granular casts.

At 100 ppm, no treatment-related changes were observed in males.

In females, there were no treatment-related histopathological kidney changes at any dose level.

Following 4 weeks off-dose, there was a partial recovery of all effects seen previously.

Table 5.3.2- 19: Kidney findings – Number of animals affected

Finding / Severity	Dose level (ppm)			
	0	**100**	**1,400**	**20,000**
Males				
Accumulation of hyaline droplets in the proximal tubule				
After 13 weeks:				
- minimal	1	0	0	0
- slight	5	0	0	0
- moderate	0	2	1	1
- severe	0	0	9	7
Total	**6/10**	**2/10**	**10/10**	**8/10**
After off-dose period				
- minimal	1	-	-	1
- slight	4	-	-	3
- moderate	2	-	-	2
Total	**7/10**	**-**	**-**	**5/10**
Single cell death – proximal tubule epithelium				
After 13 weeks:				
- minimal	5	0	0	1
- slight	0	2	1	1
- moderate	0	0	9	6
Total	**5/10**	**2/10**	**10/10**	**8/10**
After off-dose period				
- minimal	4	-	-	2
- slight	2	-	-	2
Total	**6/10**	**-**	**-**	**4/10**
Foci of basophilic (regenerating) tubules				
After 13 weeks:				
- minimal	6	3	0	1
- slight	0	3	4	7
- moderate	0	0	6	0
Total	**6/10**	**6/10**	**10/10**	**8/10**
After off-dose period				
- minimal	7	-	-	3
- slight	0	-	-	5
Total	**7/10**	**-**	**-**	**8/10**
Granular casts				
After 13 weeks:				
- minimal	0	0	1	1
- slight	0	0	0	3
Total	**0/10**	**0/10**	**1/10**	**4/10**
After off-dose period				
- Not present	10	-	-	10

No statistical analyses were performed on histopathological findings.
- : Dose levels not analysed during recovery phase (off-dose period)

III. Conclusion

The NOAEL in the 90-day dietary study in rats was 100 ppm (7.4 or 8.4 mg/kg bw/day in male and female rats respectively) based on treatment-related haematological (reduced haemoglobin and haematocrit in male rats), clinical chemistry (increased cholesterol in male rats at 1,400 ppm), urinalysis (increased urine volume and specific gravity in females at ≥ 1,400 ppm), organ weight (increase in relative liver and kidney weight in male rats and decreased of relative spleen weight in females at 1,400 ppm) and histopathological changes in the liver and kidneys at 1,400 ppm (equivalent 109 or 119 mg/kg bw/day in males and females respectively).

Following a 4-week off-dose period, there was a full or partial recovery of all treatment-related changes.

Assessment and conclusion by applicant:

Study was conducted according to OECD TG 408 and is valid and acceptable to investigate the short-term oral toxicity of fluopicolide in the rat. A NOAEL of 100 ppm (equivalent to 7.4 and 8.4 mg/kg bw/d in males and females) was determined from this study.

Data Point:	KCA 5.3.2/02
Report Author:	[redacted]
Report Year:	2006
Report Title:	AE C638206 - 90-Day toxicity study in the mouse by dietary administration
Report No:	SA 00363
Document No:	M-205579-02-1
Guideline(s) followed in study:	EEC Directive 92/69/EEC, Annex V, Method B26 (1992); JMAFF 59 NohSan 4200 (1985); OECD: 408 (1998); US-EPA OPPTS 870.3100 (1998)
Deviations from current test guideline:	Deviations from the current OECD guideline (408, 2018): - Ophthalmological examinations were not conducted - Sensory reactivity to stimuli were not performed - Blood/plasma samples were not taken for possible analysis of thyroid hormones - At termination, an evaluation of oestrus cycle was not performed - Prostate and seminal vesicles with coagulating glands were not weighed at termination Although performed in the rat, rather than the mouse, ophthalmoscopy and sensory reactivity to stimuli assessments were performed in the 90-day rat study (Mallyon, B. A.; 2000; M-197622-01-1) at doses of up to 20,000 ppm (1,671 mg/kg bw/day, combined sexes), whilst seminal vesicles with coagulating glands were weighed at termination in the rat two-generation study (Blee, M. A. B.; 2003; M-232532-01-1) as was an evaluation of oestros cycles at dose levels of up to 2,000 ppm (equivalent to approx. 100 mg/kg bw/day in F0 females throughout the 10-week pre-mating period and during gestation and lactation). Therefore, these deviations do not affect the overall acceptability of the study
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

Fluopicolide (batch number OP2050046; a fine, beige coloured powder, 95.9% purity) was administered continuously via the diet to groups of C57BL/6JICO mice (10/sex/group) at concentrations of 50, 200, 800 and 3,200 ppm (equivalent to 0, 10.4, 37.8, 161 & 770 mg/kg bw/d in males and 0, 12.6, 52.8, 207 and 965 mg/kg bw/d in females) for at least 90 days. A similarly constituted group of 10 males and 10 females received untreated diet and acted as a control. This additional 90-day mouse study was planned and performed as dose-range finder for the mouse carcinogenesis study with the same strain of mice (C57BL/6).

There were four animals found dead or sacrificed moribund between Day 6 and 73, one male from each of the dose groups 0, 200 and 3,200 ppm and one female at 800 ppm. In addition, one male at 0 ppm, two males at 800 ppm and one male at 3,200 ppm, died due to accidental trauma between Day 21 and 85. On Day 94, one control female and one female at 50 ppm died during anaesthesia. All the unscheduled deaths were considered to be incidental and not related to treatment. There were no treatment-related clinical signs.

Overall body weight gain was slightly reduced in both males (-7%) and females (-14%) at 3,200 ppm. The effect on body weight gain was most marked during Week 1 for males (-88%; statistically significant) and during Week 1 and 2 for females (-87% at Week 1, -85% at Week 2; statistically significant). Absolute body weight after one week of treatment (Day 8) was statistically significantly reduced at the high dose level by 10% and 7% in males and females, respectively. At study termination (Day 90), the reduction was only 3% in both sexes.

There was no clear treatment-related effect on food consumption.

Clinical chemistry assessment revealed a slight decrease in total cholesterol concentration in both sexes at 3,200, 800 and 200 ppm. A very slight decrease in albumin concentration was observed in both sexes at 3,200 and 800 ppm. Mean alkaline phosphatase activity was increased in males at 3,200 ppm.

Terminal body weight was slightly, but statistically significantly reduced for males at 3,200 ppm (-5%). Mean absolute and relative liver weights were increased in a dose-related manner, in both sexes at 3,200 and 800 ppm compared to control.

Macroscopic examination of decedent animals revealed no treatment-related changes. At the terminal sacrifice dark liver was noted in 4/8 males and 9/10 females at 3,200 ppm. At microscopic examination, diffuse centrilobular hepatocellular hypertrophy was observed in one decedent male at 3,200 ppm and one decedent female at 800 ppm, as well as in surviving in all high dose animals and the majority of animals at 800 ppm. The severity ranged from slight in one animal to mild in the reminder of animals at 3,200 ppm and slight in all animals at 800 ppm from the terminal sacrifice.

At the request of the Japanese Food Safety Commission, additional Proliferating Cell Nuclear Antigen (PCNA) staining on selected liver slides was conducted. The result of this additional evaluation showed that there was no relevant change in hepatocytic cell cycling for males or females at 3,200 ppm fluopicolide, when compared to controls.

The No Observed Adverse Effect Level in this 90-day dietary study in mice was 50 ppm (equivalent 10.4 and 12.8 mg/kg bw/day in males and females, respectively) based on apparent significant treatment-related reduction in cholesterol suggestive of impaired liver function at ≥ 200 ppm (equivalent 37.8 and 52.8 mg/kg bw/day in males and females, respectively). The liver was the main target organ in the study as indicated by reduced albumin and cholesterol levels, increased relative liver weights and centrilobular hepatocyte hypertrophy observed at dose levels of ≥ 800 ppm.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 technical (fluopicolide)
Purity:	98.9%
Batch no.:	OP2050046

2. Vehicle and/or positive control

Vehicle:	Diet

3. Test animals

Species:	Mice
Strain:	C57BL/6JICO
Age:	5 weeks old
Weight at start:	12.7 to 19.5 g for males and 11.6 to 16.6 g for females
Source:	[redacted]
Acclimation period:	Yes
Diet:	Certified rodent powder diet "M 20 controle" (Pietrement, Provins, France)
Water:	Water ad lib

Housing:	Mice were housed in suspended stainless-steel wire mesh cages, in groups of 3 on the day of arrival until Day 6 of the pre-study, when they were housed individually
Temperature:	20 – 24 °C
Humidity:	40% - 70%
Air changes:	10 to 15 air changes per hour (average, not monitored)
Photoperiod:	12 hours

B. Study design

1. In-life dates: September 6th 2000 to March 15th 2001

2. Animal assignment and treatment

The rats were randomized and assigned to the following test groups.

Table 5.3.2- 20: Study design

Group no.	Dose (ppm)	Number of males	Number of females
		Treatment phase	
1	0	10	10
2	50	10	10
3	200	10	10
4	800	10	10
5	3,200	10	10

On Study Days 91, 92, 93 or 94, all surviving animals from all groups were sacrificed by exsanguination under deep anesthesia (pentobarbital, intraperitoneal injection of 60 mg/kg body weight). An approximately equal number of animals randomly distributed amongst all groups were sampled on each day. Animals were fasted overnight prior to sacrifice.

3. Diet preparation and analysis

The test substance was incorporated into the diet to provide the required concentrations. The test substance was ground to a fine powder before being incorporated into the diet by dry mixing. Diet formulations were prepared twice during the course of the study, for each concentration. When not in use the diet formulations were stored at below -15 °C.

The homogeneity and stability of the test substance in the diet were demonstrated in the pre-study mix of 25 and 10,000 ppm for stability where samples of this diets were kept either at ambient temperature up to 56 days or frozen for 7 weeks at below -15 °C and ambient temperature for 1 week. The homogeneity of fluopicolide in the diet was verified for the lowest and highest concentrations from study diet mix 1 to demonstrate adequate formulation procedures. Dietary levels of the test substance were verified for each concentration.

Homogeneity of diet formulations with fluopicolide was found to be acceptable at concentrations of 25, 50, 3,200 and 10,000 ppm. Fluopicolide was found to be stable for a 7-week frozen period at below -15 °C and one week at ambient temperature. At 10,000 ppm, fluopicolide was also stably stored at ambient temperature for at least 56 days but the stability at 25 ppm stored at ambient temperature was poorer than for frozen storage. All concentration checks were in a range of 94% to 104% of the nominal values.

4. Statistics

The following variables were analysed:

- body weight parameters
- food consumptions
- clinical chemistry parameters
- organ weight parameters
- organ/body weight ratios
- organ/brain weight ratios

Statistical analysis:

Means and standard deviations (STD) were calculated for each sex separately for each group at each time period. Results of the clinical pathology and organ weight parameters were inter-compared for the treated and control groups by use of:

- Bartlett's test for homogeneity of variances between groups.
- When Bartlett's test indicated homogeneous variances, any significant differences were identified by using the combination of Analysis of Variance (ANOVA) and Dunnett's test.
- When Bartlett's test indicated heterogeneous variances, any significant differences were identified by using a modified t-test.

For body weight and food intake data Dunnett's test was used.

All tests were performed at 5% and 1% levels. All calculations and statistical analysis were performed with Xybion PathTox system (version 4.2.2).

C. Methods

1. Observations

All animals were checked for moribundity and mortality twice daily (once daily on weekends or public holidays). Observed clinical signs were recorded at least once daily for all animals. Detailed physical examinations were performed at least weekly during the treatment period. The nature, onset, severity, reversibility and duration of clinical signs were recorded. Cages and cage-trays were inspected daily for evidence of ill-health such as blood or loose faeces.

2. Body weight and food intake

Each animal was weighed once during the acclimatization period, on the first day of test substance administration, then at weekly intervals throughout the treatment period and before necropsy.

The weight of food supplied and of that remaining at the end of the food consumption period was recorded weekly for all animals during the treatment period. The mean achieved dosage intake in mg/kg bw/day for each week and for Weeks 1 to 13 was calculated.

3. Laboratory investigations (clinical chemistry)

On Study Days 91, 92, 93 or 94, in the morning, prior to necropsy, blood samples were taken from all animals in all groups by puncture of the retro-orbital venous plexus. An approximately equal number of animals randomly distributed amongst all groups were sampled on each day. Animals were fasted overnight prior to bleeding and anesthetized by inhalation of isoflurane. Blood (0.6 mL) was collected on lithium heparin for plasma chemistry determinations.

4. Biochemistry

Any significant change in the general appearance of the plasma was recorded. Total bilirubin, urea, total protein, albumin and total cholesterol concentrations and aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase activities were assayed using a Hitachi 911 (Roche Diagnostics, Meylan, France).

5. Sacrifice and pathology

On Study Days 91, 92, 93 or 94, all surviving animals from all groups were sacrificed by exsanguination under deep anaesthesia (pentobarbital, intraperitoneal injection of 60 mg/kg body weight). An approximately equal number of animals randomly distributed amongst all groups were sampled on each day. Animals were fasted overnight prior to sacrifice.

All animals, either found dead or killed by design, were necropsied. The necropsy included the examination of all major organs, tissues and body cavities. Macroscopic abnormalities were recorded and sampled and examined microscopically.

Organ weights:

Brain, heart, kidneys, liver, spleen, thymus, adrenal gland and testes were weighed fresh at scheduled sacrifice only. Paired organs were weighed together.

Tissue sampling:

The following organs and tissues from all animals were sampled:

Table 5.3.2- 21: Tissues taken for histopathology examinations

Adrenal gland	Ovary
Aorta	Pancreas
Articular surface (femoro-tibial)	Pituitary gland
Bone (sternum)	Prostate
Bone marrow (sternum)	Sciatic nerve
Brain	Seminal vesicle
Epididymis	Skeletal muscle
Esophagus	Skin
Eye and optic nerve	Spinal cord (cervical, thoracic, lumbar)
Gallbladder	Spleen
Harden an gland	Stomach
Heart	Submaxillary (salivary) gland
Intestine (duodenum, jejunum, ileum, cecum, colon, rectum)	Testis
	Thymus
Kidney	Thyroid (with parathyroid)
Larynx	Tongue
Liver	Trachea
Lung	Urinary bladder
Lymph nodes (submaxillary, mesenteric)	Uterus (including cervix)
Mammary gland	Vagina

A bone marrow smear was prepared from one femur, stained with May-Grünwald Giemsa, but not examined.

6. Histopathology

Tissue processing:

Tissue samples were fixed by immersion in neutral buffered 10% formalin with the exception of the eye, optic nerve, Harderian gland, epididymis and testis that were fixed in Davidson's fixative. Tissues were sent to Propath U.K. Ltd., Willow Court, Netherwood Road, Hereford, HR2 6JU, England for slide preparation.

With the exception of larynx, all the above-mentioned samples were embedded in paraffin wax.

Histological sections, stained with hematoxylin and eosin, were prepared for all the organs from all the animals in the control and high dose groups as well as from all decedent animals in intermediate dose groups: additionally, sections from the liver, lung, kidney, adrenal gland and thyroid gland and from gross findings observed at necropsy were prepared for all the animals in all intermediate dose groups.

Microscopic examination:

Histopathological examinations were performed on all the tissues from all the animals in the control and high dose groups and all decedents in all groups. The liver, lung, kidney, adrenal gland and thyroid gland were examined in all the animals in the study. Macroscopic findings were also examined in all intermediate dose groups.

Following the initial histopathological examination by the study pathologist, a review pathologist undertook an independent "peer review" of representative slides and diagnoses. The diagnoses presented in this report represent the consensus opinion of the two pathologists.

7. Proliferating cell nuclear antigen (PCNA) analysis

A section from a formalin-fixed paraffin-embedded block containing 2 liver samples and one duodenum sample was prepared (the duodenum has a high proliferation rate and serves as a positive staining control). The immunohistochemical reaction included incubation with a monoclonal antibody raised against PCNA, an amplification with a secondary biotinylated antibody and a streptavidin-horseradish peroxidase complex, a detection of the complex with the chromogen diamino-benzidine (DAB) and a Hematoxylin counterstaining. The immunohistochemical staining for PCNA and determination of the labeling index were performed on all surviving animals in the control group and the Group 5 (3200 ppm fluopicolide). The zonal labeling index, expressed as the number of PCNA-positive hepatocytes per thousand, was measured separately on random fields comprising at least 1000 centrilobular and 1000 periportal cells using an automatic image analysis system (technical sheet included in the study file). The mean and standard deviation were calculated for each zone and each group.

II. Results and Discussion

A. Results

1. Clinical results

There were no treatment-related signs during the course of the study.

One male from each of the dose groups 0, 200 and 3,200 ppm and one female dosed at 800 ppm were found dead or moribund between Day 6 to 73. These deaths were considered to be incidental and not treatment-related. In addition, one male at 0 ppm, two males at 800 ppm and one male at 3,200 ppm died as a result of accidental trauma between Day 21 and 85. One control female and one female at 50 ppm died during anesthesia prior to blood sampling on Day 94.

2. Body weight and food intake

Body weight

At 3,200 ppm, overall body weight gain (Day 1-90) was reduced by 7% in males and 14% in females compared to controls. The effect on body weight gain was most marked during Week 1 for males (-88%; statistically significant) and during Week 1 and 2 for females (-87% at Week 1, -85% at Week 2; statistically significant). Absolute body weight after one week of treatment (Day 8) was statistically significantly reduced at the high dose level by 10% and 7% in males and females, respectively. At study termination (Day 90), the reduction was only 3% in both sexes.

At 800 ppm, there was an apparent overall decrease in body weight gain of 14% in males (Day 1-90). However, since this effect does not occur in a dose-related manner and is not reflected in terms of absolute weight, it is thought to be attributable to the premature deaths of the two smallest animals in this group. Therefore, this apparent effect is not considered to be treatment-related. At this dose level, no effect on body weight development was observed in females.

At 200 and 50 ppm, no effect on body weight development was observed in either sex.

Table 5.3.2- 30: Mean body weight and body weight gain (g)

	Dose level (ppm)				
	0	**50**	**200**	**800**	**3,200**
	Males				
Body weight [g] *(% difference to control)*					
Day 1	17.0	17.1 *(+0.6)*	17.3 *(+1.8)*	16.9 *(-0.6)*	17.3 *(+1.8)*
Day 8	19.4	18.9 *(-2.6)*	19.6 *(+1.0)*	19.4 *(±0.0)*	17.4** *(-10.3)*
Day 15	21.0	20.7 *(-1.4)*	21.2 *(+1.0)*	20.7 *(-1.4)*	18.9** *(-10.0)*
Day 22	21.9	21.8 *(-0.5)*	21.9 *(±0.0)*	21.4 *(-2.3)*	20.0** *(-8.7)*
Day 29	22.6	22.1 *(-2.2)*	22.3 *(-1.3)*	22.0 *(-2.7)*	20.7* *(-8.4)*
Day 36	22.9	22.8 *(-0.4)*	23.0 *(+0.4)*	22.2 *(-3.1)*	21.2** *(-7.4)*
Day 43	22.9	22.9 *(±0.0)*	23.1 *(+0.9)*	22.1 *(-3.5)*	21.4** *(-6.6)*
Day 50	24.1	24.2 *(+0.4)*	24.5 *(+1.7)*	23.5 *(-2.6)*	23.2 *(-3.7)*
Day 57	24.3	24.4 *(+0.4)*	24.8 *(+2.1)*	23.7 *(-2.5)*	23.4 *(-3.7)*
Day 64	24.5	24.5 *(±0.0)*	25.0 *(+2.0)*	24.0 *(-2.0)*	23.7 *(-3.3)*
Day 71	24.8	24.9 *(+0.4)*	25.1 *(+1.2)*	24.3 *(-2.0)*	24.4 *(-1.6)*
Day 78	25.6	25.2 *(-1.6)*	25.2 *(-1.6)*	24.6 *(-3.9)*	24.6 *(-3.9)*
Day 85	26.1	26.0 *(-0.4)*	26.0 *(-0.4)*	24.8 *(-5.0)*	25.0# *(-4.2)*
Day 90	26.1	26.0 *(-0.4)*	25.9 *(-0.8)*	25.2 *(-3.4)*	25.3 *(-3.1)*

	Dose level (ppm)				
	0	50	200	800	3,200
Body weight gain [g] *(% difference to control)* +					
Day 1-8	2.5	1.8 *(-28.0)*	2.2 *(-12)*	2.5 *(±0.0)*	0.3** *(-88.0)*
Day 1-15	4.1	3.6 *(-12.2)*	3.8 *(-7.3)*	3.8 *(-7.3)*	1.9** *(-53.7)*
Day 1-22	5.0	4.7 *(-6.0)*	4.6 *(-8.0)*	4.2 *(-16.0)*	3.0* *(-40.0)*
Day 1-90	9.0	8.9 *(-1.1)*	8.5 *(-5.6)*	7.7 *(-14.4)*	8.4 *(-6.7)*
Females					
Body weight [g] *(% difference to control)*					
Day 1	14.6	15.0 *(+2.7)*	15.0 *(+2.7)*	14.4 *(-1.4)*	14.9 *(+2.1)*
Day 8	16.1	16.5 *(+2.5)*	16.5 *(+2.5)*	16.3 *(+1.2)*	15.0* *(-6.8)*
Day 15	16.6	17.6 *(+6.0)*	17.4 *(+4.8)*	16.5 *(-0.6)*	15.1* *(-9.0)*
Day 22	17.8	18.6 *(+4.5)*	18.3 *(+2.8)*	17.2 *(-3.4)*	16.1* *(-9.6)*
Day 29	18.8	19.6 *(+4.3)*	19.3 *(+2.7)*	18.6 *(-1.1)*	17.2** *(-8.5)*
Day 36	19.1	20.0 *(+4.7)*	19.5 *(+2.1)*	18.9 *(-1.0)*	17.8** *(-6.8)*
Day 43	19.4	19.9 *(+2.6)*	19.3 *(-0.5)*	19.0 *(-2.1)*	17.9** *(-7.7)*
Day 50	20.5	21.0 *(+2.4)*	20.6 *(+0.5)*	20.3 *(-1.0)*	19.3** *(-5.9)*
Day 57	20.1	21.0* *(+4.5)*	20.8 *(+3.5)*	20.4 *(+1.5)*	19.5 *(-3.0)*
Day 64	19.9	20.7 *(+4.0)*	20.5 *(+3.0)*	20.1 *(+1.0)*	19.3 *(-3.0)*
Day 71	20.5	21.4* *(+4.4)*	20.8 *(+1.5)*	20.6 *(+0.5)*	19.8 *(-3.4)*
Day 78	19.8	21.3 *(+7.6)*	21.2# *(+7.1)*	20.8 *(+5.1)*	19.9 *(+0.5)*
Day 85	20.9	21.5 *(+2.9)*	21.3 *(+1.9)*	21.0 *(+0.5)*	20.3 *(-2.9)*
Day 90	21.2	21.7 *(+2.4)*	21.7 *(+2.4)*	21.0 *(-0.9)*	20.5 *(-3.3)*
Body weight gain [g] *(% difference to control)* +					
Day 1-8	1.5	1.5 *(+0.0)*	1.5 *(+0.0)*	1.9 *(+26.7)*	0.2* *(-86.7)*
Day 1-15	2.0	2.6 *(+30.0)*	2.4 *(+20.0)*	2.0 *(±0.0)*	0.3** *(-85.0)*
Day 1-22	3.1	3.6 *(+16.1)*	3.3 *(+6.5)*	2.8 *(-9.7)*	1.3** *(-58.1)*
Day 1-90	6.6	6.7 *(+1.5)*	6.7 *(+1.5)*	6.7 *(+1.5)*	5.7 *(-13.6)*

* / ** $p < 0.05$ / $p < 0.01$, statistically different to control using Dunnet's test
# / ## $p < 0.05$ / $p < 0.01$, statistically different to control using modified t-test
+ Mean absolute body weight gains according to calculation within study report

Food intake

Due to the excessive amount of food spillage in this study, an accurate assessment of food consumption could not be made for every time interval. However, there does not appear to be a clear treatment-related effect on food consumption.

The mean achieved dietary intake of fluopicolide (Weeks 1-13) is shown in Table 5.3.2- 22.

Table 5.3.2- 22: Mean achieved dietary intake of fluopicolide (weeks 1-13)

	Dose level (ppm)							
	Males				Females			
	50	200	800	3,200	50	200	800	3,200
Concentration [mg/kg bw/day]	10.4	37.8	161	770[a]	12.6	52.8	207	965[b]

[a] No data recorded for weeks 5 and 7 due to spillage
[b] No data recorded for weeks 5, 6, 7 and 9 due to spillage

3. Laboratory investigations

Clinical chemistry:

Lower mean total cholesterol and albumin concentrations were observed in both sexes at 3,200 and 800 ppm, lower cholesterol concentrations were also noted in both sexes at 200 ppm. Mean alkaline phosphatase activity was higher in males at 3,200 ppm, only. The amplitude and statistical significance relative to controls were as follows:

Table 5.3.2- 23: Selected biochemistry results *(% difference to control)*

	Dose level (ppm)				
	0	**50**	**200**	**800**	**3,200**
Males					
Total cholesterol (mmol/L)	1.82	1.47 *(-19.2)*	1.36** *(-25.3)*	0.95** *(-47.8)*	0.91** *(-50.0)*
Albumin (g/L)	38	37 *(-2.6)*	36 *(-5.3)*	33** *(-13.2)*	33** *(-13.2)*
ALP (IU/L)	69	71 *(+2.9)*	69 *(+0.0)*	72 *(+4.3)*	89** *(+29.0)*
Females					
Total cholesterol (mmol/L)	1.51	1.43 *(-5.3)*	1.19** *(-21.2)*	1.16** *(-23.2)*	1.27** *(-15.9)*
Albumin (g/L)	38	37 *(-2.6)*	35 *(-7.9)*	34** *(-10.5)*	33** *(-13.2)*
ALP	99	102 *(+3.0)*	104 *(+5.1)*	109 *(+10.1)*	106 *(+7.1)*

** p< 0.01, significantly different from control using the Bartlett's test

4. Sacrifice and pathology

Necropsy:

Dark liver was observed in 4/8 males and in 9/10 females at 3,200 ppm.

Organ weights:

Mean terminal body weight was slightly, but statistically significantly lower (-5%) in males at 3,200 ppm when compared to controls.

Absolute liver weight was increased by 20% in males and 25% in females at 3,200 ppm, with relative liver weight increased by 30% in males and 34% in females. At 800 ppm, both absolute and relative liver weights were slightly increased by 10-14% for males and 13-16% for females at 800 ppm.

At 200 and 50 ppm, no effect on absolute and or relative liver weight was observed in either sex.

Table 5.3.2- 24: Mean absolute and relative liver weights (% difference to control)

Parameter	Dose level (ppm)									
	0	50		200		800		3,200		
Males										
Terminal BW (g)	22.3	22.3	*(±0.0)*	22.2	*(-0.4)*	21.4	*(-4.0)*	21.2*	*(-4.9)*	
Absolute liver weight (g)	1.0	1.0	*(±0.0)*	1.0	*(±0.0)*	1.1	*(+10.0)*	1.2**	*(+20.0)*	
Relative liver weight (% of body weight)	4.3	4.3	*(±0.0)*	4.5	*(+4.7)*	4.9**	*(+14.0)*	5.6**	*(+30.2)*	
Females										
Terminal BW (g)	17.7	18.0	*(+1.7)*	17.7	*(±0.0)*	17.7	*(±0.0)*	17.4	*(-1.7)*	
Absolute liver weight (g)	0.8	0.8	*(±0.0)*	0.8	*(±0.0)*	0.9*	*(+12.5)*	1.0**	*(+25.0)*	
Relative liver weight (% of body weight)	4.4	4.3	*(-2.3)*	4.7	*(+6.8)*	5.1**	*(+15.9)*	5.9**	*(+34.1)*	

* p< 0.05 ; ** p< 0.01, significantly different from control using the Bartlett's test
BW: Body weight

6. Histopathology

Unscheduled deaths:

The decedent animals showed a range of incidental changes similar to those seen at terminal sacrifice. No cause of death could be determined, except for male KT1M2422 from the control group, which showed a suppurative encephalitis, considered to be contributary to the death.

The liver from animals KT5M2502 (3,200 ppm) and KT4F2496 (800 ppm) showed a diffuse centrilobular hepatocellular hypertrophy.

Terminal sacrifice:

A treatment-related change was only seen in the liver. There was an increase in the incidence and severity (slight to mild) of diffuse centrilobular hepatocellular hypertrophy in all animals at 3,200 ppm and in a proportion of animals at 800 ppm.

All other changes were considered to be incidental and unrelated to treatment.

Table 5.3.2- 25: Liver findings – Number of animals affected

Findings / Severity	Dose level (ppm)									
	Males					Females				
	0	50	200	800	3,200	0	50	200	800	3,200
Number of animals examined	8	10	9	8	8	9	9	10	9	10
Hypertrophy, hepatocellular, centrilobular, diffuse										
-slight	0	0	0	4	1	0	0	0	8	0
-mild	0	0	0	0	7	0	0	0	0	10
Total	**0**	**0**	**0**	**4**	**8**	**0**	**0**	**0**	**8**	**10**

7. Proliferating cell nuclear antigen (PCNA)

When compared to controls, there was no relevant change in hepatocytic cell cycling in either males or females dosed at 3,200 ppm.

Table 5.3.2- 26: PCNA measurements (mean ± SD)

Zone	Dose level (ppm)			
	Males		Females	
	0	3,200	0	3,200
Number of animals examined	8	8	9	10
Centrilobular	1.63 ± 1.35	2.21 ± 0.99	1.45 ± 0.60	1.37 ± 1.01
Perilobular	2.08 ± 1.72	1.68 ± 1.19	3.03 ± 2.02	3.22 ± 1.13
Total	**1.86 ± 1.29**	**1.93 ± 0.73**	**2.23 ± 1.24**	**2.22 ± 0.97**

SD: standard deviation

III. Conclusion

The No Observed Adverse Effect Level (NOAEL) in this 90-day dietary study in mice was 50 ppm (equivalent 10.4 and 12.6 mg/kg bw/day in males and females, respectively) based on apparent significant treatment-related reduction in cholesterol suggestive of impaired liver function at ≥ 200 ppm (equivalent 37.8 and 52.8 mg/kg bw/day in males and females, respectively). The liver was the main target organ in the study as indicated by reduced albumin and cholesterol levels, increased relative liver weights and centrilobular hepatocyte hypertrophy observed at dose levels of ≥ 800 ppm (equivalent to 161 and 207 mg/kg bw/d in males and females respectively).

Assessment and conclusion by applicant:

Study was conducted in accordance with OECD TG 408 and is valid and acceptable to investigate the short-term oral toxicity of fluopicolide in mice. A NOAEL of 50 ppm (equivalent to 10.4 and 12.6 mg/kg bw/d in males and females) was determined from this study.

Data Point:	KCA 5.3.2/05
Report Author:	[redacted]
Report Year:	2000
Report Title:	AE C638206 - Mouse 90-day dietary toxicity study
Report No:	C008604
Document No:	M-197623-01-1
Guideline(s) followed in study:	US-EPA OPPTS 870.3100 (1998); OECD 408 (1998); EEC Commission Directive 88/302/EEC, Annex V, Part B; JMAFF (1985 9
Deviations from current test guideline:	Method: Deviations from current guideline SANCO/3029/99 rev. 4: There is no linearity data presented and the accuracy and precision data are determined from the procedural recoveries in the toxicological studies. However, there are at least 14 recoveries at the LOQ level and at the highest fortification level with mean accuracies between 70-110% and RSD < 20%. Considering that this analytical method is validated in support of toxicological cold studies, the method validation is considered fit for purpose. Study: Deviations from the current OECD guideline (408, 2018): - Ophthalmological examinations were not conducted - Sensory reactivity to stimuli were not performed - Blood/plasma samples were not taken for possible analysis of thyroid hormones - At termination, an evaluation of oestrus cycle was not performed - Prostate and seminal vesicles with coagulating glands were not weighed at termination Although performed in the rat, rather than the mouse, ophthalmoscopy and sensory reactivity to stimuli assessments were performed in the 90-day rat study (Mallyon, B. A.; 2000; M-197622-01-1) at doses of up to 20,000 ppm (1,671 mg/kg bw/day, combined sexes), whilst seminal vesicles with coagulating glands were weighed at termination in the rat two-generation study (Blee, M. A. B.; 2003; M-232532-01-1) as was an evaluation of oestrous cycles at dose levels of up to 2,000 ppm (equivalent to 100-200 mg/kg bw/day in F0/F1 females). Therefore, these deviations do not affect the overall acceptability of the study.
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

Groups of 10 male and 10 female Crl: CD-1 (ICR) BR mice, aged approx. 28 days, were fed diet containing 0, 32, 320, 3,200 or 6,400 ppm (equivalent to 0, 4.7, 46, 461 or 944 mg/kg bw/day in males, and 0, 6.2, 60, 629 or 1,239 mg/kg bw/day in females) fluopicolide for 13 weeks. No mortalities occurred and no clinical signs of toxicity were seen. At 6,400 ppm, overall body weight gain was reduced by 20% and 32% for males and females, respectively. At 6,400 ppm, alanine aminotransferase activity was slightly increased in both sexes, slight increases in aspartate aminotransferase and alkaline phosphatase activity were observed in males and slight increases in cholesterol and creatinine concentration in females. At 6400 ppm, absolute liver weights in males and females were increased by 42% and 60%, respectively, and relative liver weights by 50% in males and 78% in females.

At necropsy, abnormal areas in the liver were seen in 2/10 males and 3/10 females, and enlarged liver was recorded for 1/10 males and 1/10 females of the highest dose group. At 6,400 ppm, microscopic examination revealed centrilobular hepatocyte hypertrophy in the livers of 10/10 males and 10/10 females, scored as slight to severe in males and slight to moderate in females. Minimal to moderate focal hepatocyte necrosis was also seen in 3/10 males and 2/10 females. At the same dose level, 6,400 ppm, females had a 32% reduction in body weight gain over the duration of the study, whilst males had a reduction of 20%.

At 3,200 ppm, overall body weight gain was reduced by 22% for females when compared to controls. There was a slight increase in alanine aminotransferase activity in both sexes and a slight increase in aspartate aminotransferase for males only. Absolute liver weights in males and females were increased by 33% and 44%, respectively and relative liver weights by 36% in males and 59% in females.

At necropsy, abnormal areas were noted in the livers of 3/10 males and 3/10 females and an enlarged liver was seen in 1/10 females. Microscopic examination revealed minimal to moderate centrilobular hepatocyte hypertrophy in 10/10 males and 9/10 females, slight focal hepatocyte necrosis was seen in 2/10 females only.

At 320 ppm, microscopic examination revealed minimal to slight centrilobular hepatocyte hypertrophy in 9/10 males, and minimal hypertrophy in 2/10 females. There were no treatment-related effects in either sex at 32 ppm.

Thus, the No Observed Effect Level (NOEL) was 32 ppm, equivalent to a daily intake of 4.7 and 6.2 mg/kg bw/day for males and females, respectively. Since the only finding at 320 ppm was an adaptive minimal to slight centrilobular hepatocyte hypertrophy, the No Observed Adverse Effect Level (NOAEL) is considered 320 ppm, equivalent to a daily intake of 46 and 60 mg/kg bw/day for males and females, respectively and 53 mg/kg bw/day for the combined sexes.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 technical (fluopicolide)
Purity:	96.9% (10 June 1999), 97.3% (16 August 1999)
Batch no.:	Code number AE C638206 00 1C99 0005

2. Vehicle and/or positive control

Vehicle:	Diet

3. Test animals

Species:	Mice
Strain:	Crl: CD-1 (ICR) BR
Age:	Approx. 28 days old
Weight at start:	19.3 to 35.3 g (males), 18.6 to 25.3 g (females)
Source:	[redacted]
Acclimation period:	Yes
Diet:	Modified SQC Expanded Ground Rat and Mouse Maintenance Diet No. 1, supplied by Special Diet Services Ltd., Stepfield, Witham, Essex
Water:	Tap water ad lib
Housing:	Groups of five, by sex and dose group
Temperature:	19-23 °C
Humidity:	45-65%
Air changes:	Not given
Photoperiod:	12 hours

B. Study design

1. In-life dates: August 3 to November 5, 1999

2. Animal assignment and treatment

The animals were randomized and assigned to the following test groups.

After 13 weeks of continuous dietary exposure, the animals were killed.

Table 5.3.2- 27: Study design

Group no.	Dose (ppm)	Number of males	Number of females
1	0	10	10
2	32	10	10
3	320	10	10
4	3,200	10	10
5	6,400	10	10

3. Diet preparation and analysis

Test diets were prepared weekly throughout the study. On each occasion, for each dose level, a pre-mix was made using a food processor. This in turn was then mixed with remaining laboratory rodent diet and blended in a turbula mixer to produce the required dietary concentration. Subsequent concentrations were prepared by serial dilution.

Every week, aliquots of each concentration of the freshly prepared test diets (i.e. 32, 320, 3,200 and 6,400 ppm) were submitted for analysis of the test material concentration. Samples were received in powder form in plastic bags and stored deep frozen from time of receipt.

The mean results for the test diet samples analysed were within the range 93.5 - 107.0% of nominal (acceptable range +10% to -15% of nominal), except for week 9 at the 32 ppm level which gave a mean result of 111.5% of nominal. This deviation was not considered to affect the interpretation of the study.

Stability was shown to be satisfactory in previous studies over the time of use of the diet (8 days) in the concentration range 6 – 20,000 ppm i.e. % nominal values declined by a maximum of 12% over 8 days storage at room temperature.

4. Statistics

The significance of differences between control and treated groups was analysed by either parametric or non-parametric statistical tests as appropriate. A maximum 2-tailed probability value of 5% ($p < 0.05$) was considered statistically significant.

The following convention has been used to indicate statistical significance:

* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$

C. Methods

1. Observations

Observations were made twice each day, once in the morning and once in the afternoon from Mondays to Fridays except on public and Company holidays. More frequent observations were made as necessary.

Detailed observations were conducted once weekly prior to weighing. Records of observations included the nature, time of onset, severity and duration of any abnormal clinical sign/behaviour.

2. Body weight and food intake

The weight of about 20% of the animals was recorded at receipt. Each animal was weighed at randomisation, at the start of treatment, at weekly intervals thereafter and at necropsy.

For each sex, the amount of food consumed for each cage of animals was measured at weekly intervals throughout the treatment period.

3. Laboratory investigations (clinical chemistry)

Blood for haematology and biochemistry analyses was obtained from the retro-orbital sinus of each animal under isoflurane anaesthesia. Samples for haematology and clinical chemistry were collected during Study Weeks 13 and 14, respectively.

The parameters listed below were measured or derived:

<u>Haematology:</u>

Haematocrit (HCT)
Haemoglobin (HB)
Red blood cells (RBC)
Mean cell volume (MCV)
Mean cell haemoglobin (MCH)
Mean cell haemoglobin concentration (MCHC)
Platelets (PLT)
White blood cells (WBC)
Neutrophils (NEUT)
Lymphocytes (LYMP)
Monocytes (MONO)
Eosinophils (EOS)
Basophils (BASO)
Large unstained cells (LUC)
Reticulocyte count (RET)

<u>Biochemistry:</u>

Total protein (PROT)
Albumin (ALB)
Total globulin (GLOB)
A/G ratio (A/G)
Calcium (CA)
Phosphate (PO4)
Sodium (NA)
Potassium (K)
Urea (UREA)
Creatinine (CREA)
Total cholesterol (CHOL)
Total bilirubin (TBIL)
Chloride (CL)
Aspartate aminotransferase (AST)
Alanine aminotransferase (ALT)
Alkaline phosphatase (AP)
G-glutamyl transpeptidase (GGT)
Creatine kinase (CPK)
Glucose (GLUC)

4. Sacrifice and pathology

Animals were killed by exsanguination via the abdominal aorta under ether anaesthesia. All animals were examined thoroughly and macroscopic abnormalities recorded.

Organ weights:

The following organs from animals surviving to scheduled termination were weighed at necropsy:

Liver	Testes	Brain	Thymus
Kidneys	Ovaries	Adrenals	Uterus
Spleen	Epididymides	Heart	

Tissue sampling:

The following organs and tissues from all animals were fixed in 10% neutral buffered formalin, except eyes which were fixed in Davidson's fluid:

Adrenals	Liver	Seminal vesicles
Aorta	Lungs (inflated)	Skeletal muscle
Brain	Lymph nodes (cervical + mesenteric)	Skin + subcutis
Caecum		Spinal cord (3 levels)
Colon	Mammary gland	Spleen
Diaphragm	Sternum	Stomach
Duodenum	Optic nerve	Testes
Epididymides	Ovaries	Thymus
Eyes	Oviducts	Thyroid (with parathyroid)
Femur, joint	Pancreas	Tongue
Gall bladder	Pinnae[1]	Trachea
Harderian gland	Pituitary	Urinary bladder
Head[3]	Preputial gland	Uterus[2]
Heart	Prostate	Vagina
Ileum	Rectum	Any other tissue showing
Jejunum	Salivary glands (parotid, submaxillary + sublingual)	macroscopic abnormalities
Kidneys		
Lacrimal gland	Sciatic nerve	

[1] Taken for identification purposes only; not examined
[2] Cervix uteri and uterine horns examined
[3] Nose turbinates examined

A bone marrow smear was taken from all animals and fixed subsequently at staining.

5. Histopathology

Tissue processing

Following fixation, nominal 5µm sections of all organs and tissues from all animals from the control and high dose groups were prepared and stained with haematoxylin and eosin.

Liver sections from the low and intermediate dose groups were also examined microscopically, and additional sections of liver from all dose groups were stained with Oil-Red-O and examined for the presence of fat.

Microscopic examination

The following organs/tissues were examined histopathologically:

Adrenal cortex (left)	Ovary (left)
Adrenal cortex (right)	Ovary (right)
Adrenal medulla (left)	Oviduct (left)
Adrenal medulla (right)	Oviduct (right)
Aorta	Pancreas
Bone	Parathyroid
Bone marrow smear	Paroid gland
Brain	Pituitary
Caecum	Preputial gland
Cervical lymph node	Prostate
Cervix uteri	Rectum
Colon	Salivary gland, sublingual
Diaphragm	Sciatic nerve
Duodenum	Seminal vesicle
Epididymis (left)	Skin
Epididymis (right)	Spinal cord, cervical
Eye (left)	Spinal cord, thoracic
Eye (right)	Spinal cord, lumbar
Gall bladder	Spleen
Harderian gland	Stomach (fore)
Heart	Stomach (glandular)
Ileum	Submaxillary gland
Jejunum	Testis (left)
Joint	Testis (right)
Kidney (left)	Thymus
Kidney (right)	Thyroid (left)
Lacrimal gland	Thyroid (right)
Liver	Tongue
Liver ORO	Trachea
Lung	Turbinates
Lymph node, mesenteric	Urinary bladder
Mammary gland	Uterus (body)
Muscle, skeletal	Uterus (left horn)
Oesophagus	Uterus (right horn)
Optic nerve (left)	Vagina
Optic nerve (right)	

II. Results and Discussion

1. Clinical results

There were no treatment-related signs or deaths during the course of the study.

2. Body weight and food intake

Body weight

There were no statistically significant changes in body weight in either males or females at any dose level or time point measured during the study.

Over the duration of the study (Day 1-92) at 6,400 ppm, overall body weight gain was reduced by 20% and 32% compared to control in males and females, respectively. This effect was most severe during Week 1 of treatment (Day 1-8: -74% in males, -64% in females).
At 3,200 ppm and 320 ppm in females only, overall body weight gain was reduced by 22% and 18%, respectively.

The decreased body weight gain in females at 320 ppm is not considered toxicologically relevant, because effects on body weight itself were small and inconsistent (changes ranged from +2.3% to -5.8% compared to control).

Table 5.3.2- 28: Mean body weight and body weight gain (g)

	Dose level (ppm)				
	0	32	320	3,200	6,400
Males					
Body weight [g] *(% difference to control)*					
Day 1	24.5	25.9 *(+5.7)*	26.3 *(+7.3)*	26.0 *(+6.1)*	26.4 *(+7.8)*
Day 8	29.9	29.9 *(±0.0)*	29.2 *(-2.3)*	29.2 *(-2.3)*	27.8 *(-7.0)*
Day 15	32.1	32.8 *(+2.2)*	32.1 *(±0.0)*	31.9 *(-0.6)*	30.0 *(-6.5)*
Day 22	33.4	34.1 *(+2.1)*	33.7 *(+0.8)*	33.4 *(±0.0)*	31.6 *(-5.4)*
Day 29	34.9	36.0 *(+3.2)*	35.5 *(+1.7)*	34.8 *(-0.3)*	33.2 *(-4.9)*
Day 36	36.0	37.2 *(+3.3)*	36.3 *(+0.8)*	36.5 *(+1.4)*	34.8 *(-3.3)*
Day 43	36.8	38.0 *(+3.3)*	38.2 *(+3.8)*	36.9 *(+0.3)*	35.5 *(-3.5)*
Day 50	37.8	38.9 *(+2.9)*	39.0 *(+3.2)*	37.7 *(-0.3)*	36.0 *(-4.8)*
Day 57	39.0	40.0 *(+2.5)*	40.5 *(+3.8)*	39.0 *(±0.0)*	37.2 *(-4.6)*
Day 64	39.4	39.9 *(+1.3)*	40.6 *(+3.0)*	38.7 *(-1.8)*	37.1 *(-5.8)*
Day 71	38.9	40.7 *(+4.6)*	41.9 *(+7.7)*	39.1 *(+0.5)*	37.7 *(-3.1)*
Day 78	40.0	41.1 *(+2.8)*	42.4 *(+6.0)*	39.6 *(-1.0)*	38.6 *(-3.5)*
Day 85	41.4	41.6 *(+0.5)*	44.1 *(+6.5)*	41.1 *(-0.7)*	39.3 *(-5.1)*
Day 92	41.2	41.9 *(+1.9)*	43.6 *(+6.0)*	40.6 *(-1.2)*	39.7 *(-3.4)*
Body weight gain [g] *(% difference to control)* [+]					
Day 1-8	5.4	4.0 *(-25.9)*	2.9 *(-46.3)*	3.2 *(-40.7)*	1.4 *(-74.1)*
Day 1-15	7.6	6.9 *(-9.2)*	5.8 *(-23.7)*	5.9 *(-22.4)*	3.6 *(-47.4)*
Day 1-92	16.6	16.0 *(-3.6)*	17.3 *(+4.2)*	14.6 *(-12.0)*	13.3 *(-19.9)*

	Dose level (ppm)				
	0	32	320	3,200	6,400
Females					
Body weight [g] *(% difference to control)*					
Day 1	23.9	23.0 *(-3.8)*	23.7 *(-0.8)*	22.7 *(-5.0)*	23.8 *(-0.4)*
Day 8	25.0	24.7 *(-1.2)*	24.6 *(-1.6)*	23.8 *(-4.8)*	24.2 *(-3.2)*
Day 15	25.8	25.4 *(-1.6)*	25.3 *(-1.9)*	24.5 *(-5.0)*	25.7 *(-0.4)*
Day 22	27.4	27.0 *(-1.5)*	27.3 *(-0.4)*	26.1 *(-4.7)*	27.2 *(-0.7)*
Day 29	29.4	28.7 *(-2.4)*	28.8 *(-2.0)*	27.6 *(-6.1)*	28.5 *(-3.1)*
Day 36	29.9	29.6 *(-1.0)*	28.7 *(-4.0)*	28.1 *(-6.0)*	28.8 *(-3.7)*
Day 43	29.5	30.4 *(+1.7)*	29.7 *(+0.7)*	29.0 *(-1.7)*	29.7 *(+0.7)*
Day 50	31.3	31.3 *(±0.0)*	30.0 *(-4.2)*	28.7 *(-8.3)*	29.6 *(-5.4)*
Day 57	32.3	31.9 *(-1.2)*	31.3 *(-3.1)*	30.6 *(-5.3)*	30.7 *(-4.6)*
Day 64	30.7	31.3 *(+2.3)*	31.3 *(+2.3)*	28.9 *(-5.9)*	30.5 *(-0.7)*
Day 71	32.6	33.0 *(+1.2)*	31.7 *(-2.8)*	30.4 *(-6.7)*	30.5 *(-6.4)*
Day 78	33.8	33.0 *(-2.4)*	32.5 *(-3.8)*	30.6 *(-9.5)*	31.0 *(-8.3)*
Day 85	32.3	33.5 *(+3.7)*	32.6 *(+0.9)*	30.5 *(-5.6)*	31.0 *(-4.0)*
Day 92	34.2	33.7 *(-1.5)*	32.2 *(-5.8)*	30.7 *(-10.2)*	30.8 *(-9.9)*
Body weight gain [g] *(% difference to control)*					
Day 1-8	1.1	1.7 *(+54.6)*	0.9 *(-18.2)*	1.1 *(±0.0)*	0.4 *(-63.6)*
Day 1-15	1.9	2.4 *(+26.3)*	1.6 *(-15.8)*	1.8 *(-5.3)*	1.9 *(±0.0)*
Day 1-92	10.3	10.7 *(+3.9)*	8.5 *(-17.5)*	8.0 *(-22.3)*	7.0 *(-32.0)*

* / ** p < 0.05 / p < 0.01, statistically different to control using Dunnett's test
# / ## p < 0.05 / p< 0.01, statistically different to control using modified t-test
+ No statistical analyses were performed for body weight gains (only between each study day).

Food intake

Food consumption was not affected by treatment.

The mean achieved dietary intake of fluopicolide is shown in Table 5.3.2- 29.

Table 5.3.2- 29: Mean achieved dietary intake of fluopicolide

	Dose level (ppm)							
	Males				Females			
	32	320	3,200	6,400	32	320	3,200	6,400
Concentration [mg/kg bw/day]	4.7	46	461	944	6.2	60	629	1,239

3. Laboratory investigations

Haematology:

There were no treatment-related effects.

Clinical chemistry:

At 6,400 ppm, there was a slight increase in alanine aminotransferase activity and of creatinine in both sexes. Aspartate aminotransferase and alkaline phosphatase activity were slightly increased in males only, whilst slight increases in cholesterol concentration were observed in females.

At 3,200 ppm, there was a slight increase in alanine aminotransferase activity in both sexes, a slight increase in aspartate aminotransferase activity in males and an increase of creatinine in females.

There were no treatment-related effects at 320 or 32 ppm.

Other statistically significant differences were considered to be of no toxicological significance because they were either not dose-related, or values were within the normal range of historical controls.

Table 5.3.2- 30: Selected biochemistry results *(% difference to control)*

Parameter	Dose level (ppm)				
	0	32	320	3,200	6,400
Males					
ALT (U/L)	42	43 *(+2.4)*	48 *(+14.3)*	83** *(+97.6)*	75*** *(+78.6)*
AST (U/L)	61	78 *(+27.9)*	70 *(+14.8)*	85* *(+39.3)*	103*** *(+68.9)*
Alkaline phosphatase (U/L)	45	35 *(-22.2)*	36 *(-20.0)*	71 *(+57.8)*	93** *(+106.7)*
Cholesterol (mmol/L)	3.28	3.11 *(-5.2)*	3.53 *(+7.6)*	2.94 *(-10.4)*	2.97 *(-9.5)*
Creatinine (µmol/L)	38	37 *(-2.6)*	40 *(+5.3)*	42 *(+10.5)*	45*** *(+18.4)*
Females					
ALT (U/L)	32	33 *(+3.1)*	34 *(+6.3)*	69** *(+115.6)*	79*** *(+146.9)*
AST (U/L)	78	76 *(-2.6)*	82 *(+5.1)*	99 *(+26.9)*	86 *(+10.3)*
Alkaline phosphatase (U/L)	56	55 *(-1.8)*	75 *(+33.9)*	53 *(-5.4)*	67 *(+19.6)*
Cholesterol (mmol/L)	2.24	1.83 *(-18.3)*	2.07 *(-7.6)*	2.68 *(+19.6)*	3.11** *(+38.8)*
Creatinine (µmol/L)	34	32 *(-5.9)*	35 *(+2.9)*	42*** *(+23.5)*	48*** *(+41.2)*

* p<0.05; ** p< 0.01; *** p<0.001, statistically different to control

4. Sacrifice and pathology

Necropsy:

At 6,400 ppm, there were abnormal areas in the livers of 2/10 males and 3/10 females and enlarged livers for 1/10 males and 1/10 females.

At 3,200 ppm, there were abnormal areas in the livers of 3/10 males and 3/10 females and an enlarged liver for 1/10 females.

Organ weights:

At 6,400 ppm, absolute liver weight in males and females was increased by 42% and 60%, respectively, and liver weight relative to body weight was increased by 50% in males and 78% in females when compared with controls.

At 3,200 ppm, absolute liver weight in males and females was increased by 33% and 44%, respectively, and liver weight relative to body weight was increased by 36% in males and 59% in females when compared with controls.

There were no effects on organ weights in either sex at 320 or 32 ppm.

Other statistically significant differences were considered to be of no toxicological significance because they were either not dose-related, or values were within the normal range of historical controls.

Table 5.3.2- 31: Mean absolute and relative liver weights *(% difference to control)*

Parameter	Dose level (ppm)				
	0	32	320	3,200	6,400
Males					
Terminal body weight (g)	40.0	40.8 *(+2.0)*	42.5 *(+6.3)*	39.2 *(-2.0)*	37.8 *(-5.5)*
Absolute liver weight (g)	1.83	1.89 *(+3.2)*	2.05 *(+12.0)*	2.44** *(+33.3)*	2.60** *(+42.1)*
Relative liver weight (% of body weight)	4.57	4.63 *(+1.3)*	4.82 *(+5.5)*	6.22** *(+36.1)*	6.86** *(+50.1)*
Females					
Terminal body weight (g)	33.2	31.6 *(-4.8)*	30.9 *(-6.9)*	30.0 *(-9.6)*	30.0 *(-9.6)*
Absolute liver weight (g)	1.60	1.48 *(-7.5)*	1.61 *(+0.63)*	2.30** *(+51.3)*	2.56** *(+60.0)*
Relative liver weight (% of body weight)	4.81	4.67 *(-2.9)*	5.19 *(+7.9)*	7.65** *(+59.0)*	8.56** *(+78.0)*

* p<0.05; ** p< 0.01, statistically different to control (Dunnett´s test)

6. Histopathology

At 6,400 ppm, centrilobular hepatocyte hypertrophy was present in the livers of 10/10 males and 10/10 females, and was scored as slight to severe in males and slight to moderate in females. There was also minimal to moderate hepatocyte necrosis in 3/10 males and 2/10 females, respectively.

At 3,200 ppm, minimal to moderate centrilobular hepatocyte hypertrophy was present in the livers of 10/10 males and 9/10 females. Slight focal hepatocyte necrosis was seen in 2/10 females only.

At 320 ppm, minimal to slight centrilobular hepatocyte hypertrophy was seen in the livers of 9/10 males, and was also present at minimal severity in 2/10 females. There were no other treatment-related histopathological changes.

Table 5.3.2- 32: Liver findings – Number of animals affected

Findings / Severity	Dose level (ppm)									
	Males					Females				
	0	32	320	3,200	6,400	0	32	320	3,200	6,400
Centrilobular hepatocyte hypertrophy										
-minimal	0	1	6	1	0	1	0	2	2	0
-slight	0	0	3	5	2	0	0	0	6	7
-moderate	0	0	0	4	5	0	0	0	1	3
-severe	0	0	0	0	3	0	0	0	0	0
Total	**0/10**	**1/10**	**9/10**	**10/10**	**10/10**	**1/10**	**0/10**	**2/10**	**9/10**	**10/10**
Focal hepatocyte necrosis										
-minimal	0	0	0	0	1	0	0	0	0	0
-slight	0	0	0	1	0	1	0	0	2	2
-moderate	0	0	0	0	2	0	0	0	0	0
Total	**0/10**	**0/10**	**0/10**	**1/10**	**3/10**	**1/10**	**0/10**	**0/10**	**2/10**	**2/10**

III. Conclusion

In this study, overall body weight gain was reduced in males and females at 6,400 ppm and at 3,200 and 320 ppm in females only. However, the decreased bodyweight gain at 320 ppm in females is not considered toxicologically relevant, since is not reflected in terms of absolute body weight.

The main target organ was the liver based on increased transaminase activities, increased organ weight and adverse histopathological findings at both the 6,400 and 3,200 ppm dose levels.

The No Observed Effect Level (NOEL) was 32 ppm, equivalent to a daily intake of 4.7 and 6.2 mg/kg bw/day for males and females, respectively. Since the only finding at 320 ppm was an adaptive minimal to slight centrilobular hepatocyte hypertrophy, the No Observed Adverse Effect Level (NOAEL) is considered 320 ppm, equivalent to a daily intake of 46 and 60 mg/kg bw/day for males and females, respectively and 53 mg/kg bw/day for the combined sexes.

Assessment and conclusion by applicant:

The study was conducted according to OECD TG 408 and is valid and acceptable to investigate the short-term oral toxicity of fluopicolide in mice. A NOAEL of 32 ppm (equivalent to 4.7 and 6.2 mg/kg bw/d in males and females) was determined from this study

Data Point:	KCA 5.3.2/03
Report Author:	[redacted]
Report Year:	2000
Report Title:	AE C638206 - Dog 90-day oral toxicity study
Report No:	C010655
Document No:	M-199397-01-1
Guideline(s) followed in study:	EEC Commission Directive 88/302/EEC, Annex V, Part B (1987); JMAFF (1985); OECD 409 (1998); US-EPA OPPTS 870.1350 (1998)
Deviations from current test guideline:	Method: Deviations from current guideline SANCO/3029/99 rev. 4: There is no linearity data presented and the accuracy and precision data are determined from the test suspension samples in the toxicological study. However, there are 16 samples per concentration level with mean accuracies between 70-110% and RSD < 20%. Considering that this analytical method is validated in support of a toxicological cold study, the method validation is considered fit for purpose.; Study: Deviations from the current OECD guideline (409, 1998): none
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

Groups of 4 male and 4 female beagle dogs were treated by gavage with 0, 5, 70 or 1,000 mg/kg bw/day of fluopicolide daily for at least 13 consecutive weeks. The test substance was administered in a vehicle of 1% w/v methyl cellulose in distilled water at a constant volume of 5 mL/kg bw. Controls received the vehicle alone. Animals were observed daily for clinical signs. Body weight was recorded weekly, whilst food consumption was measured daily. A detailed clinical examination and ophthalmoscopy were carried out pre-treatment and prior to necropsy. Haematology and biochemistry investigations were conducted twice prior to treatment, during Week 7, and prior to termination. Urinalysis was conducted prior to treatment, during Week 6, and at termination. At necropsy, the weights of selected organs were recorded, and subsequently a range of tissues was examined histopathologically.

There were no mortalities or clinical signs of toxicity observed during the study. In addition, there were no treatment-related ophthalmic changes and no effect on food intake, haematology, biochemistry, urinalysis, macroscopic pathology or histopathology. There were no statistically significant effects on absolute body weight or body weight gain in either sex. However, body weight gain was reduced at the highest dose level by 27% and 33% in males and females.

At 1,000 mg/kg bw/day, absolute liver weight was increased by 19% in males and by 32% in females, compared to controls. Liver weight relative to bodyweight ratio was increased by 28% and 43% in males and females, respectively, when compared to controls. Organ weights were unaffected at 5 or 70 mg/kg bw/day.

The NOAEL in the 90-day dietary study in dogs was 70 mg/kg bw/day based on the increased absolute and relative liver weight at 1,000 mg/kg bw/day for both sexes. Dogs are noted to be a non-rodent species and the large increase in liver weight is therefore considered toxicologically relevant.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	Technical AE C638206 (fluopicolide)
Purity:	97.7%
Batch no.:	PP/241024/2 & PP/241067/1

2. Vehicle and/or positive control

Vehicle:	1% w/v methyl cellulose in distilled water

3. Test animals

Species:	Dogs
Strain:	Outbred beagle dogs
Age:	6 to 7 month old
Weight at start:	9.5-11.5 kg (males), 6.7-12.9 kg (females)
Source:	[redacted]
Acclimation period:	Yes
Diet:	Harlan Teklad 9682, supplied by Harlan Teklad Ltd., Shaw's Farm, Bicester, Oxon, UK
Water:	Water ad lib
Housing:	Housed in pairs (except during feeding and dosing, or on specific veterinary advice, when they were housed individually), by sex and dose group, under controlled environmental conditions, in a solid floor pen, measuring approximately 1 m x 4.5 m, with wood chips as bedding
Temperature:	18 ± 2 °C
Humidity:	45 - 65%
Air changes:	Not given
Photoperiod:	12 hours

B. Study design

1. In-life dates: November 9th 1999 to March 10th 2000

2. Animal assignment and treatment

The animals were randomized and assigned to the following test groups:

Table 5.3.2- 33: Study design

Group no.	Dose (mg/kg bw/day)	Number of males	Number of females
Treatment phase			
1	0	4	4
2	5	4	4
3	70	4	4
4	1,000	4	4

Dose levels were 0, 5, 70 and 1,000 mg/kg bw/day by gavage (see Table 5.3.2- 33). Dose levels were selected on the basis of previous toxicity data in the dog.

Males were treated for at least 91 consecutive days, and females for at least 93 consecutive days.

3. Dose preparation and analysis

Prior to the start of treatment, a procedure was developed to reliably prepare homogeneous and suitably stable mixtures of the test material in the vehicle, 1% w/v methyl cellulose in distilled water, at the required nominal concentrations of 1, 14 and 200 mg/mL (equivalent to dose levels of 5, 70 and 1,000 mg/kg bw/day, respectively).

Throughout the study dosing suspensions were generally prepared one day in advance of dosing, except for dosing over the weekends, the following Mondays and bank holidays, in which instances doses for these days were prepared up to 4 days in advance.

On each occasion, the required volume of dosing mixture was prepared by gradually adding the vehicle to the appropriate quantity of test material and thoroughly mixing them. Initially, a smooth paste was prepared which was made up to volume with more vehicle and the mixture was then homogenised.

Samples from Days 1, 29, 57 and 85 of treatment were analysed at all levels. The mean results for the test suspensions analysed were 87% to 107.6% of nominal (acceptable range +20% to -20% of nominal). Homogeneity was within acceptable limits, and stability was also shown to be satisfactory after four days storage at 4 °C.

4. Statistics

The significance of differences between control and treated groups was analysed by either parametric or non-parametric statistical tests as appropriate. A maximum 2-tailed probability value of 5% ($p < 0.05$) was considered statistically significant.

The convention employed to indicate statistical significance at the levels of $p < 0.05$, $p < 0.01$ and $p < 0.001$ is indicated below the relevant tables.

C. Methods

1. Observations

Each dog was given a thorough clinical examination, including measurement of rectal temperature, prior to the start of and at the end of the treatment period.

Animals were observed daily for abnormal behaviour, including neuro-muscular coordination and physical appearance. Observations were made once each morning.

They were also checked in the afternoon on Mondays to Fridays except on public and Company holidays. More frequent observations were made as necessary.

Records of observations included the nature, time of onset, severity and duration of any abnormal clinical sign/behaviour.

2. Body weight and food intake

The weight of each dog was recorded upon receipt, at randomisation, on Study Days -14 and -7, and at the start of treatment. Thereafter, each animal was weighed weekly throughout the treatment period and at necropsy.

Food consumption for each animal was measured daily for two weeks prior to the start of the study, and then daily throughout the treatment period.

3. Ophthalmoscopy

Ophthalmological examinations were conducted by a consultant veterinarian on all dogs prior to the start of treatment. During the last week of treatment only the eyes of animals in the control and high dose groups were examined. Both eyes of each animal were examined using an indirect ophthalmoscope after instillation of 1.0% w/v Mydriacyl.

4. Laboratory investigations (clinical chemistry)

Blood samples for haematology and biochemistry were collected from the jugular vein of all animals on two occasions prior to the start of treatment (Study Days -15 and -7), and during weeks 7 and 13 of treatment. Samples for urinalysis were collected by catheterisation on Study Days -5 and during week 6 of treatment, and were taken directly from the bladder at necropsy.

The parameters listed below were measured or derived:

Haematology:

Table 5.3.2- 34: Haematology parameters

Haematocrit (HCT)	Lymphocytes (LYMP)
Haemoglobin (HB)	Monocytes (MONO)
Red blood cells (RBC)	Eosinophils (EOS)
Mean cell volume (MCV)	Basophils (BASO)
Mean cell haemoglobin (MCH)	Large unstained cells (LUC)
Mean cell haemoglobin concentration (MCHC)	Erythrocyte sedimentation rate (ESR)
Platelets (PIT)	Reticulocytes (RET)
White blood cells (WBC)	Prothrombin time (PT)
Neutrophils (NEUT)	Activated partial thromboplastin time (APTT)

Biochemistry:

Table 5.3.2- 35: Biochemistry parameters

Total protein (PROT)	Glucose (GLUC)
Albumin (ALB)	Total cholesterol (CHOL)
Total globulin (GLOB)	Total bilirubin (TBIL)
A/G ratio (A/G)	Chloride (CL)
Calcium (CA)	Aspartate aminotransferase (AST)
Phosphate (PO4)	Alanine aminotransferase (ALT)
Sodium (NA)	Alkaline phosphatase (AP)
Potassium (K)	G-glutamyl transpeptidase (GGT)
Urea (UREA)	Creatine kinase (CPK)
Creatinine (CREA)	

Urinalysis:

Table 5.3.2- 36: Urinalysis parameters

Appearance (APP)	Bacteria (BACT)
Specific gravity (SG)	Red blood cells (RBC)
Protein (PROT)	Epithelial cells (EPTH)
Bilirubin (BIL)	Phosphate crystals (PO4)
pH (PH)	Urate crystals (URAT)
Glucose (GLUC)	Casts (CAST)
Urobilinogen (UBIL)	White blood cells (WBC)
Ketones (KET)	Sperm (SPER)
Blood (BLD)	Spun deposit colour (SDEP)

4. Sacrifice and pathology

All animals were killed by exsanguination of the carotid artery whilst under deep anaesthesia induced by intravenous injection of pentobarbitone sodium. Males were killed on Study Days 92 and 93 and females on Study Days 94 and 95. Where possible, one dog was necropsied in turn from each dose group in the following sequence: Group 1, Group 4, Group 2, Group 3 where possible. Within each dose group, dogs were necropsied in animal number order.

All dogs were examined thoroughly and any macroscopic abnormalities recorded.

Organ weights:

The following organs were weighed:

Table 5.3.2- 37: Organ weights

Adrenals	Prostate
Brain	Spleen
Epididymides	Testes
Heart	Thymus
Kidneys	Thyroid
Liver	Uterus + oviducts
Ovaries	
Pituitary	

Tissue sampling:

The following organs and tissues from all animals were fixed intact or sampled and fixed in 10% neutral buffered formalin, except eyes which were fixed in Davidson's fluid:

Table 5.3.2- 38: Tissue sampling

Adrenals	Larynx[3]	Sciatic nerve
Articulated surface and shaft of femur	Liver	Skeletal muscle
	Lungs	Skin + subcutis
Aorta	Lymph nodes (mandibular, mesenteric)	Spinal cord (3 levels)
Brain		Spleen
Caecum	Mammary gland	Sternum
Colon	Nose[3]	Stomach
Diaphragm	Oesophagus	Testes
Duodenum	Optic nerve	Thymus
Epididymides	Ovaries + oviducts	Thyroid + parathyroid
Eyes	Pancreas	Tongue
Gall bladder	Pharynx[3]	Tonsils
Head[3]	Pinnae[1]	Trachea
Heart	Pituitary	Urinary bladder
Ileum	Prostate	Uterus[2]
Jejunum	Rectum	Vagina
Kidneys	Salivary glands (parotid, mandibular + sublingual)	Any other tissue showing macroscopic abnormalities
Lacrimal gland		

[1] The ear with tattoo and ID implant was taken for identification purposes only and was not examined.
[2] Cervix uteri and uterine horns examined.
[3] The head, pharynx, larynx and nose were taken but not examined. Bone marrow smears were taken from the 7th thoracic rib of all animals and fixed at staining.

5. Histopathology

Tissue processing:

Following fixation, nominal 5 µm sections of all organs and tissues from each animal were prepared and stained with haematoxylin and eosin, except eyes (Davidson's fixative) and bone marrow smears (fixed with Wright's stain). An additional frozen section of the liver from all animals was stained with Oil Red O to evaluate the presence of fat.

Microscopic examination:

Tissues were examined for histopathological change with a light microscope. The data were entered directly onto a computer terminal using the Roclee Pathology system which then generated the summary tables and individual animal reports.

II. Results and Discussion

A. Results

1. Clinical results

There were no mortalities or treatment-related clinical signs.

2. Body weight and food intake

Body weight

There were no statistically significant effects on absolute body weight or body weight gain in either sex. However, overall body weight gain (day 1-92) was reduced at the highest dose level by 27% and 33% in males and females.

Table 5.3.2- 31: Mean body weight and body weight gain (kg)

	Dose level (mg/kg bw/day)			
	0	**5**	**70**	**1,000**
		Males		
Body weight [kg] *(% difference to control)*				
Day 1	10.3	10.2 *(-0.1)*	10.2 *(-0.1)*	10.0 *(-0.3)*
Day 8	10.5	10.5 *(±0.0)*	10.4 *(-1.0)*	10.3 *(-1.9)*
Day 15	10.9	10.8 *(-0.9)*	10.7 *(-1.8)*	10.5 *(-3.7)*
Day 22	11.2	11.1 *(-0.9)*	10.9 *(-2.7)*	10.7 *(-4.5)*
Day 29	11.3	11.2 *(-0.9)*	11.1 *(-1.8)*	10.9 *(-3.5)*
Day 36	11.6	11.5 *(-0.9)*	11.3 *(-2.6)*	11.2 *(-3.4)*
Day 43	12.0	11.9 *(-0.8)*	11.6 *(-3.3)*	11.4 *(-5.0)*
Day 50	12.1	12.0 *(-0.8)*	11.7 *(-3.3)*	11.7 *(-3.3)*
Day 57	12.2	12.2 *(±0.0)*	11.8 *(-3.3)*	11.8 *(-3.3)*
Day 64	12.3	12.4 *(+0.8)*	11.7 *(-4.9)*	11.9 *(-3.3)*
Day 71	12.5	12.6 *(+0.8)*	12.1 *(-3.2)*	12.0 *(-4.0)*
Day 78	12.6	12.6 *(±0.0)*	12.1 *(-4.0)*	11.9 *(-5.6)*
Day 85	12.6	12.9 *(+2.4)*	12.1 *(-4.0)*	12.0 *(-4.8)*
Day 92	12.8	13.1 *(+2.3)*	12.2 *(-4.7)*	11.9 *(-7.0)*
Body weight gain [kg] *(% difference to control)*				
Day 1-92	2.0	2.9 *(+11.5)*	2.0 *(-23.1)*	1.9 *(-26.9)*

	Dose level (mg/kg bw/day)			
	0	5	70	1,000
Females				
Body weight [kg] *(% difference to control)*				
Day 1	8.9	9.6 *(+7.9)*	9.5 *(+6.7)*	9.1 *(+2.2)*
Day 8	9.0	9.7 *(+7.8)*	9.7 *(+7.8)*	9.1 *(+1.1)*
Day 15	9.3	9.8 *(+5.4)*	9.8 *(+5.4)*	9.1 *(-2.2)*
Day 22	9.5	9.9 *(+4.2)*	9.9 *(+4.2)*	9.2 *(-3.2)*
Day 29	9.6	10.0 *(+4.2)*	10.1 *(+5.2)*	9.5 *(-1.0)*
Day 36	9.8	10.3 *(+5.1)*	10.4 *(+6.1)*	9.6 *(-2.0)*
Day 43	10.1	10.6 *(+5.0)*	10.5 *(+4.0)*	9.9 *(-2.0)*
Day 50	10.2	10.6 *(+3.9)*	10.5 *(+2.9)*	9.9 *(-2.9)*
Day 57	10.3	10.6 *(+2.9)*	10.7 *(+3.9)*	9.9 *(-3.9)*
Day 64	10.3	10.8 *(+4.9)*	10.9 *(+5.8)*	9.9 *(-3.9)*
Day 71	10.6	10.8 *(+1.9)*	10.8 *(+1.9)*	10.1 *(-4.7)*
Day 78	10.6	11.2 *(+5.7)*	10.9 *(+2.8)*	10.2 *(-3.8)*
Day 85	10.6	11.2 *(+5.7)*	11.0 *(+3.8)*	10.2 *(-3.8)*
Day 92	10.7	11.3 *(+5.6)*	11.0 *(+2.8)*	10.3 *(-3.7)*
Body weight gain [kg] *(% difference to control)*				
Day 1-92	1.8	1.7 *(-5.6)*	1.6 *(-11.1)*	1.2 *(-33.3)*

* / ** $p < 0.05$ / $p < 0.01$, statistically different to control using Dunnet´s test
# / ## $p < 0.05$ / $p < 0.01$, statistically different to control using modified t-test

Food intake

There was no treatment-related effect on food consumption in either sex. There were also no consistent differences in food conversion ratios between treated and control groups.

Table 5.3.2- 32: Mean food consumption (g/animal/day) *(% difference to control)*

	Dose level (mg/kg bw/day)			
	0	5	70	1,000
Males				
Week 1	390	398 *(+2.1)*	401 *(+2.8)*	397 *(+1.8)*
Week 2	393	400 *(+1.8)*	389 *(-1.0)*	400 *(+1.8)*
Week 3	378	393 *(+4.0)*	396 *(+4.8)*	388 *(+2.6)*
Week 4	391	400 *(+2.3)*	400 *(+2.3)*	400 *(+2.3)*
Week 5	390	400 *(+2.6)*	391 *(+0.3)*	398 *(+2.1)*
Week 6	398	400 *(+0.5)*	400 *(+0.5)*	397 *(-0.3)*
Week 7	392	401 *(+2.3)*	400 *(+2.0)*	384 *(-2.0)*
Week 8	396	401 *(+1.3)*	400 *(+1.0)*	382 *(-3.5)*
Week 9	371	401 *(+6.9)*	396 *(+5.6)*	380 *(+1.3)*
Week 10	395	400 *(+1.3)*	387 *(-2.0)*	379 *(-4.1)*
Week 11	377	400 *(+6.1)*	385 *(+2.1)*	368 *(-2.4)*
Week 12	390	401 *(+2.8)*	400 *(+2.6)*	368 *(-5.6)*
Week 13	400	400 *(±0.0)*	394 *(-1.5)*	388 *(-3.0)*

	Dose level (mg/kg bw/day)			
	0	5	70	1,000
Females				
Week 1	330	358 *(+8.5)*	383 *(+16.1)*	298 *(-10.0)*
Week 2	342	361 *(+5.6)*	384 *(+12.3)*	332 *(-2.9)*
Week 3	343	344 *(+0.3)*	341 *(-0.6)*	313 *(-9.6)*
Week 4	356	359 *(+0.8)*	382 *(+7.3)*	345 *(-3.1)*
Week 5	363	376 *(+3.6)*	387 *(+6.6)*	348 *(-4.1)*
Week 6	369	383 *(+3.8)*	381 *(+3.3)*	344 *(-6.8)*
Week 7	362	376 *(+3.9)*	359 *(-0.8)*	330 *(-8.8)*
Week 8	372	382 *(+2.7)*	386 *(+3.8)*	329 *(-11.6)*
Week 9	358	375 *(+4.7)*	374 *(+4.5)*	348 *(-2.8)*
Week 10	350	378 *(+8.0)*	375 *(+7.1)*	336 *(-4.0)*
Week 11	351	381 *(+8.3)*	374 *(+6.6)*	317 *(-9.7)*
Week 12	353	366 *(+3.7)*	377 *(+6.8)*	331 *(-6.1)*
Week 13	347	377 *(+8.6)*	372 *(+7.2)*	314 *(-9.5)*

* / ** p < 0.05 / p < 0.01, statistically different to control using Dunnett's test
# / ## p < 0.05 / p< 0.01, statistically different to control using modified t-test

3. Ophthalmological results

There were no treatment-related findings.

4. Laboratory investigations

Haematology:

There were no treatment-related effects.

Clinical chemistry:

There were no treatment-related effects.

Urinalysis:

No treatment-related abnormalities in the urine were detected.

5. Sacrifice and pathology

Necropsy:

No treatment-related macroscopic abnormalities were seen.

Organ weights:

At 1,000 mg/kg bw/day, absolute liver weight was increased by 19% in males and by 32% in females, when compared to controls. Liver weight relative to body weight was increased by 28% and 43% in males and females, respectively, when compared to controls.

There was no effect on organ weights in either sex at 70 or 5 mg/kg bw/day.

Table 5.3.2- 39: Mean absolute and relative liver weights *(% difference to control)*

Parameter	Dose level (ppm)					
	0	5		70		1,000	
Males							
Terminal BW (kg)	12.74	12.88	*(+1.1)*	12.16	*(-4.5)*	11.80	*(-7.4)*
Absolute liver weight (g)	368.1	351.1	*(-0.2)*	350.6	*(-4.8)*	438.8*	*(+19.2)*
Relative liver weight (% of body weight)	2.9	2.7	*(-6.9)*	2.9	*(+0.0)*	3.7*	*(+27.6)*
Females							
Terminal BW (kg)	10.64	11.15	*(+4.8)*	11.13	*(+4.6)*	10.19	*(-4.2)*
Absolute liver weight (g)	300.1	322.3	*(+7.4)*	318.6	*(+6.1)*	396.4	*(+32.1)*
Relative liver weight (% of body weight)	2.8	2.9	*(+3.6)*	2.9	*(+3.6)*	4.0*	*(+42.9)*

* p< 0.05, significantly different from control
BW: Body weight

6. Histopathology

No treatment-related effects were observed.

Minor changes observed were considered to be spontaneous in origin and of no toxicological significance.

III. Conclusion

There were no mortalities or clinical signs of toxicity observed during the study. In addition, there were no treatment-related ophthalmic changes and no effect on body weight, food intake, haematology, biochemistry, urinalysis, macroscopic pathology or histopathology. At 1,000 mg/kg bw/day, absolute liver weight was increased by 19% in males and by 32% in females, compared to controls. Liver weight relative to bodyweight ratio was increased by 28% and 43% in males and females, respectively, when compared to controls.

Thus, the NOAEL in the 90-day dietary study in dogs was 70 mg/kg bw/day based on the increased absolute and relative liver weight at 1000 mg/kg bw/day for both sexes. Dogs are noted to be a non-rodent species and the large increase in liver weight is therefore considered toxicologically relevant.

Assessment and conclusion by applicant:

Study was conducted according to OECD TG 409 and is valid and acceptable to investigate the short-term oral toxicity of fluopicolide in dogs. A NOAEL of 70 mg/kg bw/d was determined from this study.

Data Point:	KCA 5.3.2/04
Report Author:	[redacted]
Report Year:	2002
Report Title:	AE C638206 - 52-week toxicity study by oral route (gavage) in beagle dogs
Report No:	C029194
Document No:	M-216694-01-1
Guideline(s) followed in study:	EEC 96/54/EEC B.32 (1996); JMAFF 59 NohSan No. 4200 (1985); OECD 452 (1981); US-EPA OPPTS 870.4100 (1998)
Deviations from current test guideline:	Method: Deviations from current guideline SANCO/3029/99 rev. 4: There is no calibration plot or calibration equation presented, however the calibration range and coefficients of determination (>0.9996) are reported. Considering that this analytical method is validated in support of a toxicological cold study, the method validation is considered fit for purpose.; Study: Deviations from the current OECD guideline (452, 2018): - Harderian gland, oesophagus, seminal vesicles with coagulating glands and trachea were not sampled, fixed or examined histopathologically These deviations do not affect the overall acceptability of the study.
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

A total of 40 beagle dogs (20 males and 20 females) of average body weight 8.9 kg for the males and 8.2 kg for the females at the start of the experiment, were divided into four groups of 5 animals/sex/group which received doses of 0, 70, 300 or 1,000 mg/kg bw/day of fluopicolide by the oral (gavage) route. The test substance was administered as a suspension in 1% aqueous methylcellulose solution, at a constant dosage-volume of 5 mL/kg bw/day. The control group was given the same dose-volume of the vehicle by the same route on the same occasions.

The animals were checked at least twice daily for mortality, morbidity and clinical signs were observed once a day. A detailed clinical examination was performed once prior to the start of treatment, and once weekly throughout the treatment period. The body weights were recorded at least once before the start of the study, on the first day of treatment and then at weekly intervals. Food consumption was recorded daily, starting 7 days before treatment and then throughout the treatment period. An ophthalmological examination was performed once before treatment and during Weeks 12, 25 and 51. Haematological, blood biochemical and urinalysis investigations were performed prior to the treatment period and during weeks 13, 26 and 51. On completion of treatment, the animals were sacrificed. All animals were subjected to a complete macroscopic post-mortem examination. Selected organs were weighed and specified tissues preserved. Selected tissues from all animals were examined microscopically.

At 1,000 mg/kg bw/day, the males had no body weight gains and an increased incidence of regurgitation and salivation occasions was observed. At the same dose, an increase in cholesterol levels in females in Week 51 (+42%, $p \leq 0.05$) was measured which may suggest possible impairment of some liver functions.

At 300 mg/kg bw/day also a slight increased incidence of regurgitation and salivation occasions was seen, whereas at 70 mg/kg bw/day no treatment-related effects occurred.

Gross examination at necropsy showed liver enlargement in single animals at ≥300 mg/kg bw/day. However, organ weights did not reveal any statistically significant intergroup differences. In addition, microscopic examination did not reveal any changes in organs and tissues. Therefore, the gross necropsy findings are considered adaptive and not toxicologically adverse.

In conclusion, the test item, fluopicolide caused reduced body weight gain of males and increased cholesterol levels in females at 1,000 mg/kg bw/day as the main treatment-related effects. Thus, considering that regurgitation and salivation were secondary effects due to the viscous dosage form at high concentration gavage application, the No Observed Adverse Effect Level (NOAEL) was established at 300 mg/kg bw/day.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 (fluopicolide)
Purity:	95.9%
Batch no.:	OP2050046

2. Vehicle and/or positive control

Vehicle:	1% aqueous methylcellulose solution

3. Test animals

Species:	Dogs
Strain:	Beagle dogs
Age:	6 months old
Weight at start:	8.95 kg (range: 7.90 to 10.1 kg) for the males and 8.18 kg (range: 6.70 to 10.4 kg) for the females
Source:	[redacted]
Acclimation period:	Yes
Diet:	UAR 125 C2 pelleted diet, batch Nos. 10201, 10308,10403,10509, 10530, 10716, 10731, 10629, 10927 and 11020 and from day 275 UAR 125 C3 pelleted diet, batch Nos. 11102, 11205, 20109, 20208 and 20320 (UAR, Villemoisson, Epinay-sur-Orge, France)
Water:	Water ad lib
Housing:	Individually housed in pens containing wood shavings (SICSA, Alfortville, France) for bedding material, except when a urine sample was required. The dogs were group-housed once a week by sex and group after the last recording of clinical signs (afternoon) until the next morning
Temperature:	20 ± 5 °C
Humidity:	50 ± 20%
Air changes:	12 cycles/hour of filtered, non-recycled air
Photoperiod:	12 hours

B. Study design

1. In-life dates: March 26th 2001 to April 15th 2002

2. Animal assignment and treatment

The animals were randomized and assigned to the following test groups.

Table 5.3.2- 40: Study design

Group no.	Dose (ppm)	Number of males	Number of females
Treatment phase			
1	0	5	5
2	70	5	5
3	300	5	5
4	1,000	5	5

The dosage forms were administered daily for a period of 52 weeks. Day 1 corresponds to the first day of treatment.

3. Dose preparation and analysis

The oral route was selected since it is a possible route of exposure in man.

The dosage forms were administered by gavage using a plastic syringe fitted with a plastic oesophageal tube, once a day, at approximately the same time. The quantity of dosage formulation administered to each animal was adjusted according to the most recently recorded body weight. A constant dosage-volume of 5 mL/kg bw/day was used. The dosage forms were stirred continuously throughout the dosing procedure.

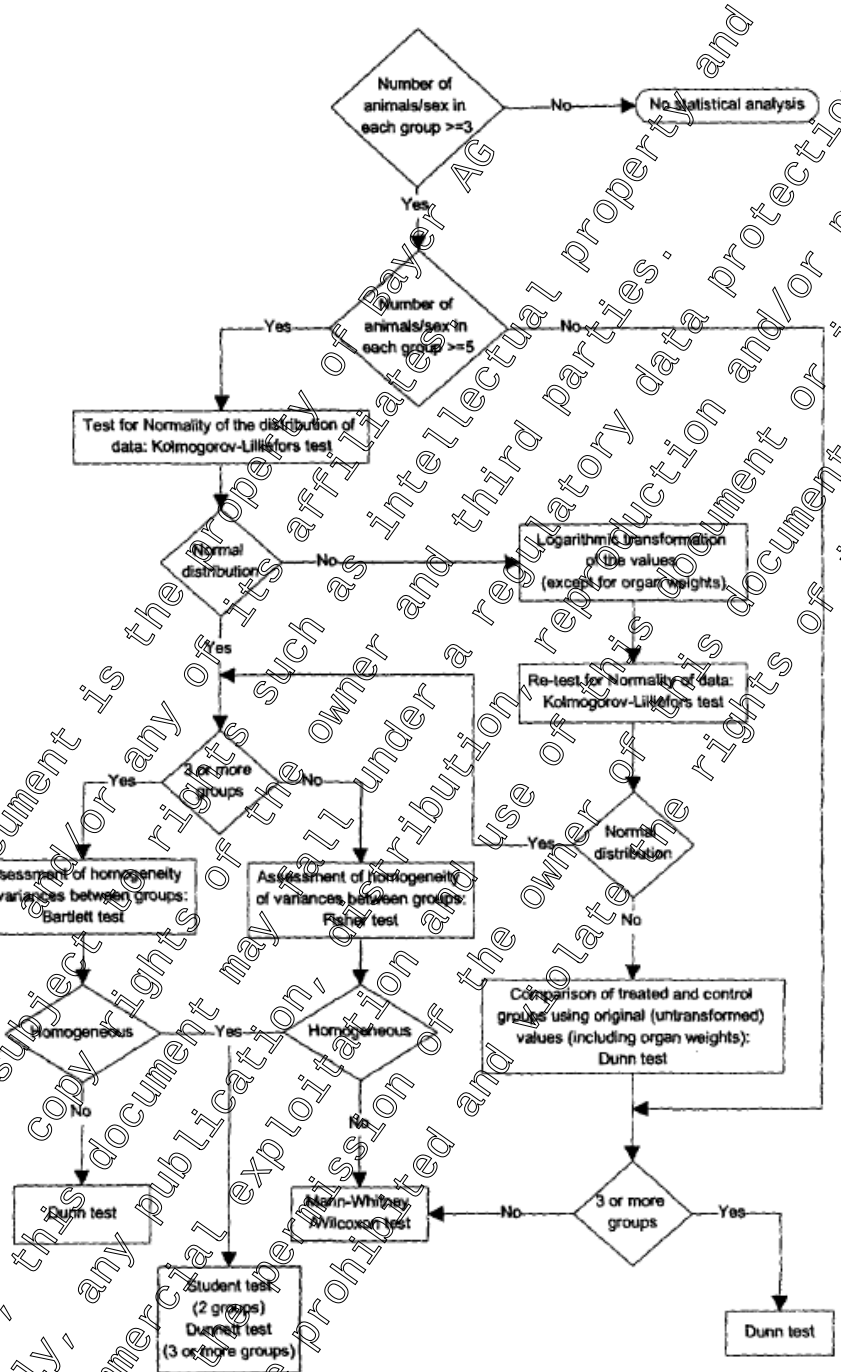
The results of the analyses demonstrated the homogeneity of each dosage form analysed before the study (14 and 200 mg/mL).

The results of the analyses demonstrated the stability of dosage forms prepared at 14 and 200 mg/mL over a six hours period at room temperature (away from light).

Throughout the study a satisfactory agreement was observed between the nominal and actual concentrations of the test item in the administered dosage forms since the deviations from nominal concentrations were in the acceptable range of ± 10%.

4. Statistics

The following sequence was used for the statistical analyses of body weight, food consumption, hematology, blood biochemistry, urinalysis and organ weight data:



C. Methods

1. Observations

Each animal was observed at least once a day, at approximately the same time, for the recording of clinical signs.

Each animal was checked at least twice a day, including during weekends and public holidays, for mortality or signs of morbidity. A macroscopic post-mortem examination was performed on the found dead female X50163 of dose group 3 and the required tissues preserved for a microscopic examination.

A detailed clinical examination was performed on all animals before the beginning of the treatment period and once weekly during the study.

These observations were made outside the kennels and included (but not limited to) evaluation of the skin, fur, eyes, mucous membranes, occurrence of secretions and excretions and autonomic activity (e.g. salivation, lacrimation, piloerection, pupil size, unusual respiratory pattern).

Changes in gait, posture, and response to handling as well as presence of clonic or tonic movements, stereotypes (e.g. excessive grooming, repetitive circling) or bizarre behaviour (e.g. self-mutilation, walking backwards) were recorded.

Due to the low weight of males of the high dose group, a detailed clinical examination was performed by a veterinarian on these animals in week 32, for health status.

2. Body weight and food intake

The body weight of each animal was recorded once before allocation of the animals to groups, on the first day of treatment, and then once a week until the end of the study.

The quantity of food consumed by each animal was recorded daily by weighing the quantity given and that remaining the next morning. Food intake per animal and per day was calculated 7-days before the beginning of the treatment period and then throughout the study. Whenever fasting was required, the food was removed at the end of the day and calculation of food consumed was made at that time (over a period of approx. 5 hours for that day).

3. Ophthalmoscopy

Ophthalmological examination was performed on all animals before the beginning of the treatment period and one occasion during Weeks 12, 25 and 51.

Pupillary light and blink reflexes were evaluated first. The pupils of the animals were then dilated with tropicamide (Mydriaticum®, Merck Sharp & Dohme-Chibret, Paris, France), and the appendages, optic media and fundus were examined by indirect ophthalmoscopy. Anterior segment and the lens, were also examined by a slit-lamp biomicroscope.

4. Laboratory investigations (clinical chemistry)

Blood samples were taken from a jugular vein without anaesthesia (before the daily treatment) and collected into tubes containing the appropriate anticoagulant (see below).

Prior to blood sampling and during urine collection, the animals were deprived of food for an overnight period of at least 14 hours. The urine was collected in the presence of thymol crystals.

Haematology:

The following parameters were determined for all animals once before the beginning of the treatment period and during weeks 13, 26 and 51. In addition, a complete haematological examination for a clinical diagnostic was performed on the male X50118 of group 4 group which presented respiration distress (abdominal breathing) and lateral decubitus on day 95 approx. 30 minutes after dosing.

Table 5.3.2- 41: Haematology parameters

Mean cell haemoglobin (MCH)	Eosinophils (E)
Thrombocytes (PLAT)	Basophils (B)
Differential white blood cell count with cell morphology[a]	Reticulocytes (RET)[b]
Neutrophils (N)	Prothrombin time (PT)
Lymphocytes (L)	Activated partial thromboplastin time (APTT)
Monocytes (M)	Fibrinogen (FIB)

(a): blood smears were prepared from all sampled animals. If the samples were not accepted by the H1 Analyzer, a microscopic evaluation was performed after May Grunwald Giemsa staining.
(b): blood smears were prepared from all sampled animals. As no anaemia was observed, the reticulocyte count was not determined.

Bone marrow:

Bone marrow smears were prepared from the sternum of all animals killed on completion of the treatment period for examination of the bone marrow and differential cell count.

As no relevant abnormalities were observed in haematological investigations, the bone marrow differential cell count was not determined.

Biochemistry:

The following parameters were determined for all animals once before the beginning of the treatment period and during weeks 13, 26 and 51. In addition, a complete blood biochemical investigation was performed on the male X50118 of group 4 for a clinical diagnostic.

Table 5.3.2- 42: Biochemistry parameters

Total protein (PROT)	Cholesterol (CHOL)
Albumin (ALB)	Triglycerides (TRIG)
A/G ratio (A/G)	Total bilirubin (TOT.BIL)
Calcium (Ca++)	Chloride (Cl-)
Inorganic phosphorus (I.PHOS)	Aspartate aminotransferase (ASAT)
Sodium (Na+)	Alanine aminotransferase (ALAT)
Potassium (K+)	Alkaline phosphatase (AP)
Urea (UREA)	G-glutamyl transferase (GGT)
Creatinine (CREAT)	Creatine kinase (CK)
Glucose (GLUC)	
Total Proteins (PROT)	

Urinalysis:

The following parameters were determined for all animals once before the beginning of the treatment period and during Weeks 13, 26 and 51.

Table 5.3.2- 43: Urinalysis parameters

Appearance (APP) Colour (COLOUR) Volume (VOLUME) Specific gravity (SP.GRAV) pH (pH) Protein (PROT) Bilirubin (BILI) Glucose (GLUC) Urobilinogen (UROB) Ketones (CETO) Blood (BLOOD)	Cytology of sediment: Leucocytes (WBC) Erythrocytes (RBC) Cylinders (CYLIN) Magnesium ammonium phosphate crystals (AMM.PH) Calcium phosphate crystals (CAL.PH) Calcium oxalate crystals (CAL.OX) cells (CELLS)

4. Sacrifice and pathology

Macroscopic examination:

On completion of the treatment period, after at least 14 hours fasting, all surviving animals were anesthetized by an intravenous injection of thiopental sodium and killed by exsanguination.

A complete macroscopic post-mortem examination was performed on all study animals. This included examination of the external surfaces, all orifices, the cranial cavity, the external surface of the brain and spinal cord, the thoracic, abdominal and pelvis cavities with their associated organs and tissues and the neck with its associated organs and tissues.

Organ weights:

The body weight of all animals killed at the end of the treatment period was recorded before sacrifice, and the organs specified in the Tissue Procedures Table were weighed wet as soon as possible after dissection.

The ratio of organ weight to body weight (recorded immediately before sacrifice) and the ratio of organ weight to brain weight were calculated.

Table 5.3.2- 44: Organ weights

Adrenals Brain Epididymides Heart Kidneys Liver	Ovaries Spleen Testes Thyroids with parathyroid Uterus (horn and cervix)

Tissue sampling:

For all study animals, the tissues specified in the Tissue Procedures Table were preserved in 10% buffered formalin (except for the eyes with optic nerve which were fixed in Davidson's fixative and the testes and epididymides which were preserved in Bouin's fluid).

Table 5.3.2- 45: Tissue sampling

Macroscopic lesions	Lungs with bronchi	Spinal cord (cervical, thoracic and lumbar)
Adrenals	Lymph nodes (mandibular, mesenteric)	Spleen
Aorta	Mammary glands/area	Sternum with bone marrow
Brain	Nasal cavities	Stomach
Caecum	Optic nerve	Testes
Colon	Ovaries	Thymus
Duodenum	Pancreas	Thyroids with parathyroid
Epididymides	Pharynx	Tongue
Eyes	Pituitary	Trachea
Femoral bone with articulation	Prostate	Urinary bladder
Gall bladder	Rectum	Uterus (horn and cervix)
Heart	Salivary glands (parotid and submandibular)	Vagina
Ileum		
Jejunum		
Kidneys	Sciatic nerve	
Larynx	Skeletal muscle	
Liver	Skin	

5. Histopathology

Tissue processing:

All tissues required for microscopic examination were embedded in paraffin wax, sectioned at thickness of approximately four microns and stained with haematoxylin-eosin. This work was performed at Histotox (La Rochelle, France).

In addition, Oil red O stainings were performed on frozen sections of liver of all animals from the control and high dose groups killed at the end of the treatment period and for the female that died.

Microscopic examination:

A microscopic examination was performed on all tissues listed in Table 5.3.2- 46 for all study animals.

Table 5.3.2- 46: Microscopic examination

Macroscopic lesions	Lymph nodes (mandibular, mesenteric)	Spinal cord (cervical, thoracic and lumbar)
Adrenals	Mammary glands/area	Spleen
Aorta	Nasal cavities	Sternum with bone marrow
Brain	Optic nerve	Stomach
Caecum	Ovaries	Testes
Colon	Pancreas	Thymus
Duodenum	Pharynx	Thyroids with parathyroid
Epididymides	Pituitary	Trachea
Eyes	Prostate	Urinary bladder
Gall bladder	Rectum	Uterus (horn and cervix)
Heart	Salivary glands (parotid and submandibular)	Vagina
Ileum	Sciatic nerve	
Jejunum	Skeletal muscle	
Kidneys	Skin	
Larynx		
Liver		
Lungs with bronchi		

II. Results and Discussion

A. Results

1. Clinical results and Mortality

There was no treatment-related mortality during the study. One female (X50163) of the 300 mg/kg bw/day dose group died. No specific indices for death were identified. After a review of clinical signs prior to death and macroscopic and microscopic post-mortem examinations, accidental aspiration of stomach contents into the lungs after regurgitation was suggested by the investigators as a possible cause of death.

Excessive salivation, vomiting and regurgitation were the most frequently observed clinical signs in treated animals. All these signs were seen transiently and sometimes noted among control animals.

The slightly higher incidence of excessive salivation recorded in the 1,000 mg/kg bw/day groups (3/5 males on 12 occasions; 3/5 females on 34 occasions) was considered to be due to the viscous dosage form at that high concentration (200 mg/mL). Male X50108 of this group presented respiration distress (abdominal breathing) and lateral decubitus on day 95 approx. 30 minutes after dosing. This was considered to be caused by difficult administration of the viscous dosage form at that concentration leading to regurgitation and probable accidental aspiration of stomach contents into the lungs. A relationship to toxicity of the test item was ruled out.

Table 5.3.2- 47: Clinical observations

Finding	Dose level (mg/kg bw/day)							
	Males				Females			
	0	70	300	1,000	0	70	300	1,000
Number of animals examined	5	5	5	5	5	5	5	5
Excessive salivation	0	1	2	3	2	1	2	3
Abdominal breathing	1	0	0	1	1	0	0	0
Lateral decubitus	0	0	0	1	0	0	0	0
Vomiting								
normal	0	0	1	0	0	0	0	0
colourless	1	0	0	0	0	1	0	0
whitish	0	0	1	1	0	1	0	0
yellowish	1	0	0	0	0	0	0	0
brown	0	0	0	1	0	1	0	0
brownish	1	0	1	2	0	0	0	2
Regurgitation	0	1	3	2	1	0	1	1

The other recorded clinical signs were occasional, of low incidence, noted in control animals, without obvious dose-relationship and/or commonly noted in beagle dogs. Therefore, they were not considered to be related to treatment.

Detailed clinical examination:

No abnormalities were noted in any group. Males given 1,000 mg/kg bw/day were in healthy condition as observed in week 52.

Consequently, there were no treatment-related clinical signs in any groups.

2. Body weight and food intake

Body weight

The body weight development of males given 70 or 300 mg/kg bw/day was similar to that of controls. Treatment with the test item at 1,000 mg/kg bw/day affected the body weight gain of males: after 52 weeks of treatment, males given 1,000 mg/kg bw/day did not gain any weight, and few animals (X50116, X50119 and X50120) had body weight loss on some occasions (values of body weight loss varying from -0.4 to -1.2 kg).

The body weight gain of females treated at 70, 300 and 1,000 mg/kg bw/day was not affected by treatment with the test item.

Table 5.3.2- 48: Mean body weight and body weight gain (kg)

	Dose level (mg/kg bw/day)			
	0	**70**	**300**	**1,000**
Males				
Body weight [kg] *(% difference to control)*				
Week -3	8.7	8.8 *(+1.2)*	8.8 *(+1.2)*	8.7 *(±0.0)*
Week 1	9.0	8.9 *(-1.1)*	9.1 *(+1.1)*	8.8 *(-2.2)*
Week 4	8.9	9.1 *(+2.2)*	9.2 *(+3.4)*	8.9 *(±0.0)*
Week 8	9.2	9.5 *(+3.3)*	9.5 *(+3.3)*	8.7 *(-5.4)*
Week 12	9.4	9.8 *(+4.3)*	9.7 *(+3.2)*	9.0 *(-4.3)*
Week 16	9.5	10.0 *(+5.3)*	9.9 *(+4.2)*	8.9 *(-6.3)*
Week 20	9.5	10.0 *(+5.3)*	9.8 *(+3.2)*	8.8 *(-7.4)*
Week 24	9.5	10.2 *(+7.4)*	10.1 *(+6.3)*	8.8 *(-7.4)*
Week 28	9.6	10.3 *(+7.3)*	10.1 *(+5.2)*	8.7 *(-9.4)*
Week 32	9.5	10.4 *(+9.5)*	10.0 *(+5.3)*	8.7 *(-8.4)*
Week 36	9.5	10.4 *(+9.5)*	10.1 *(+6.3)*	8.7 *(-8.4)*
Week 40	9.8	10.7 *(+9.2)*	10.4 *(+6.1)*	8.9 *(-9.2)*
Week 44	9.9	10.8 *(+9.1)*	10.4 *(+5.1)*	8.9 *(-10.1)*
Week 48	10.0	10.9 *(+9.0)*	10.5 *(+5.0)*	9.1 *(-9.0)*
Week 52	9.8	10.6 *(+8.2)*	10.5 *(+7.1)*	8.8 *(-10.2)*
Body weight gain [kg] *(% difference to control)* [+]				
Week 1-52	0.8	1.7 *(+112.5)*	1.4 *(+75.0)*	0.0 *(-100.0)*
Females				
Body weight [kg] *(% difference to control)*				
Week -3	8.0	7.6 *(-5.0)*	7.8 *(-2.5)*	7.7 *(-3.7)*
Week 1	8.4	8.0 *(-4.8)*	8.3 *(-1.2)*	8.0 *(-4.8)*
Week 4	8.4	8.1 *(-3.6)*	8.4 *(±0.0)*	8.2 *(-2.4)*
Week 8	9.1	8.6 *(-5.5)*	9.1 *(±0.0)*	8.9 *(-2.2)*
Week 12	9.2	8.7 *(-5.4)*	9.3 *(+1.1)*	9.1 *(-1.1)*
Week 16	9.5	8.9 *(-6.3)*	9.4 *(-1.1)*	9.3 *(-2.1)*
Week 20	9.5	9.1 *(-4.2)*	9.4 *(-1.1)*	9.5 *(±0.0)*
Week 24	9.7	9.0 *(-7.2)*	9.3 *(-4.1)*	9.3 *(-4.1)*
Week 28	9.5	9.2 *(-3.2)*	9.6 *(+1.1)*	8.9 *(-6.3)*
Week 32	9.3	9.1 *(-2.2)*	9.5 *(+2.2)*	9.0 *(-3.2)*
Week 36	9.2	9.2 *(±0.0)*	9.6 *(+4.3)*	8.8 *(-4.3)*
Week 40	9.6	9.5 *(-1.0)*	10.0 *(+4.2)*	9.1 *(-5.2)*
Week 44	9.4	9.4 *(±0.0)*	9.7 *(+3.2)*	9.2 *(-2.1)*
Week 48	9.6	9.3 *(-3.1)*	9.7 *(-1.0)*	9.4 *(-2.1)*
Week 52	9.7	9.7 *(±0.0)*	10.1 *(+4.1)*	9.3 *(-4.1)*
Body weight gain [kg] *(% difference to control)* [+]				
Week 1-52	1.3	1.7 *(+30.8)*	1.8 *(+38.5)*	+1.3 *(±0.0)*

* / ** $p < 0.05$ $p < 0.01$, statistically different to control using Dunnet´s test
[+] No statistical analyses were performed for body weight gains

Food intake

Statistically significant changes in food consumption were recorded occasionally throughout the study in both males and females. However, since the effects were inconsistent and the overall amount of food consumed by treated animals was comparable to controls, these are considered to be unrelated to treatment.

Table 5.3.2- 49: Mean food consumption (g) *(% difference to control)*

Parameter	Dose level (mg/kg bw/day)			
	0	70	300	1,000
Males				
Week 1-52	299	292 *(-2.3)*	293 *(-2.0)*	291 *(-2.7)*
Females				
Week 1-52	282	275 *(-2.5)*	263 *(-6.7)*	262 *(-7.1)*

No statistical analyses were performed for mean food consumption for the whole study period

3. Ophthalmological results

There were no treatment-related findings.

4. Laboratory investigations

Haematology:

When compared to the mean control or pre-dose values, there were no treatment-related differences in the haematological parameters in any group.

Clinical chemistry:

Slightly increased cholesterol levels were noted in females given 1,000 mg/kg bw/day in Week 51, as indicated in Table 5.3.2- 50.

Some differences occasionally attaining statistical significance were noted in treated animals when compared with controls. However these were not considered to be related to treatment with the test item since they were slight, not necessarily dose-related and/or individual values were within the range of the laboratory historical background data.

Table 5.3.2- 50: Cholesterol data (mean ± SD)

	Dose level (mg/kg bw/day)							
	Males				Females			
	0	70	300	1,000	0	70	300	1,000
Cholesterol concentration (mmol/L)								
Pre-dose	3.0± 0.5	3.2± 0.7	2.9± 0.6	2.6± 0.5	3.2± 0.4	2.8± 0.2	2.8± 0.1	2.9± 0.3
Week 13	2.9± 0.4	3.6± 0.6	3.4± 0.6	3.3± 1.1	3.5± 0.9	3.8 ± 0.9	4.6 ± 1.3	5.0 ± 1.6
Week 26	3.0± 0.6	3.6± 0.5	3.3± 0.7	2.8± 0.9	3.8 ± 0.4	4.4 ± 1.6	3.8 ± 0.7	4.4 ± 0.8
Week 51	2.9± 0.6	3.5± 0.8	3.5± 0.6	3.6± 2.1	3.8 ± 0.7	4.4 ± 1.2	3.9 ± 0.7	5.4*± 0.8

* / ** p < 0.05 / p < 0.01, statistically different to control
SD: standard deviation
Historical control range for plasma cholesterol level in females: 2.3-4.8 mmol/L

Urinalysis:

There were no changes in any qualitative or quantitative urinary parameters that could be attributed to treatment with the test item.

The increased urine volume noted in treated males during week 51 was not considered to be of toxicological importance since this difference was due to the abnormal low values of control males at this period.

5. Sacrifice and pathology

Necropsy:

Animals found dead:

At necropsy of the female (X50163) found dead in group 3, the main findings were foamy contents in the trachea, dilated/coloured lungs together with several greyish/whitish area, reddish liquid contents hi the thoracic cavity and dilated stomach with yellowish liquid contents.

Terminal sacrifice:

The liver was enlarged in 1/5 males and 1/5 females given 300 mg/kg bw/day and in 3/5 males given 1,000 mg/kg/day. The kidneys were enlarged in 1/5 males given 300 mg/kg bw/day. As these necropsy findings were not associated with relevant histopathological changes, they were considered to be of no toxicological adversity.

The few other macroscopic findings noted were those which are commonly encountered in the untreated laboratory beagle dog kept under laboratory condition and thus were considered to be unrelated to treatment.

Organ weights:

Some changes in relative liver, thyroid and spleen weights to body weight or brain weight were observed in treated dogs compared to controls. However, they are considered to be of no toxicological significance since the differences were slight, without statistical significance and without any relevant histopathological changes.

The other differences noted in organ weights were minor, without any dose-relationship, and/or without the same trend in the two sexes. They were therefore not considered to be related to treatment.

Table 5.3.2- 51: Selected absolute and relative organ weights *(% difference to control)*

Parameter	Dose (mg/kg bw/day)			
	0	70	300	1,000
Males				
Liver				
Absolute weight (g)	303.8	298.2 *(-1.8)*	336.8 *(+10.9)*	354.3 *(+16.6)*
Relative to body weight (%)	3.08	2.82 *(-8.4)*	3.20 *(+3.9)*	4.01 *(+30.2)*
Relative to brain weight (%)	413.4	422.9 *(+2.3)*	458.7 *(+11.0)*	489.2 *(+18.3)*
Spleen				
Absolute weight (g)	29.46	28.91 *(-1.9)*	26.71 *(-9.3)*	23.99 *(-18.6)*
Relative to body weight (%)	0.296	0.267 *(-9.8)*	0.255 *(-13.9)*	0.273 *(-7.8)*
Relative to brain weight (%)	40.21	40.91 *(+1.7)*	36.33 *(-9.6)*	33.12 *(-17.6)*
Thyroid				
Absolute weight (g)	0.826	0.857 *(+3.8)*	0.871 *(+5.4)*	0.804 *(-2.7)*
Relative to body weight (%)	0.0081	0.0079 *(-2.5)*	0.0082 *(+1.2)*	0.0092 *(+13.6)*
Relative to brain weight (%)	1.13	1.22 *(+8.0)*	1.19 *(+5.3)*	1.11 *(-1.8)*
Females				
Liver				
Absolute weight (g)	261.5	279.9 *(+7.0)*	279.0 *(+6.7)*	273.1 *(+4.4)*
Relative to body weight (%)	2.74	3.01 *(+9.8)*	2.91 *(+6.2)*	3.06 *(+11.7)*
Relative to brain weight (%)	400.8	378.4 *(-5.6)*	385.9 *(-3.7)*	384.6 *(-4.0)*
Spleen				
Absolute weight (g)	28.02	32.09 *(+14.5)*	31.92 *(+13.9)*	24.89 *(-11.2)*
Relative to body weight (%)	0.291	0.351 *(+20.6)*	0.330 *(+13.4)*	0.278 *(-4.5)*
Relative to brain weight (%)	43.11	43.12 *(+0.02)*	44.09 *(+2.3)*	35.12 *(-18.5)*
Thyroid				
Absolute weight (g)	0.756	0.808 *(+6.9)*	0.798 *(+5.6)*	0.866 *(+14.6)*
Relative to body weight (%)	0.0079	0.0086 *(+8.9)*	0.0082 *(+3.8)*	0.0097 *(+22.8)*
Relative to brain weight (%)	1.16	1.09 *(-6.0)*	1.11 *(-4.3)*	1.23 *(+6.0)*

6. Histopathology

Unscheduled death:

The major microscopic findings in the found dead female (X50163) were marked desquamative interstitial pneumonitis, moderate foamy alveolar macrophages and subpleural fibrosis in the lungs, adrenal cortical cell hypertrophy and vascular ectasia (agonic) in the intestines and some internal organs and tissues. No factor contributing to the death could be clearly established.

Terminal sacrifice:

No treatment-related microscopic findings were noted.

All microscopic changes recorded were recognized as those commonly observed in the Beagle dog kept under laboratory conditions. Moreover, they were present with approximately similar incidence and severity in both control and treated animals.

In addition, the microscopic examination of the frozen liver sections stained with Oil red O, showed that except for one male dog from the top dose group (1,000 mg/kg bw/day) where strong positive reaction for Oil red O was found, in all other animals examined very weak to moderate positive reactions were noted with approximately similar incidence in both control and treated animals. Consequently, it was concluded that the treatment with the test item did not exert an effect on lipid deposition/distribution in the liver.

III. Conclusion

The liver appeared to be the main target organ in the one year oral toxicity study in dogs and was characterised by adaptive enlargement of the liver. Increased plasma cholesterol levels may suggest possible impairment of some liver functions. The NOAEL in the one year oral toxicity study was 300 mg/kg bw/day based on impaired body weight gain in males and slight increased cholesterol levels in females at 1,000 mg/kg bw/day.

Assessment and conclusion by applicant:

The study was conducted according to OECD 452 and is valid and acceptable to determine the short-term oral toxicity of fluopicolide (1-year) in the dog. A NOAEL of 300 mg/kg bw/d was determined from this study.

CA 5.3.3 Other routes

Data Point:	KCA 5.3.3/01
Report Author:	[redacted]
Report Year:	2003
Report Title:	A subacute dermal toxicity study in rats with AE C638206
Report No:	C036489
Document No:	M-220782-01-1
Guideline(s) followed in study:	US-EPA OPPTS 870.3200 (1998)
Deviations from current test guideline:	Deviations from the current OECD guideline (410, 1981): None.
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

Fluopicolide was administered dermally under a semi-occlusive covering to male and female Wistar rats five days/week for four weeks. The dose groups for this study were 0 (control group), 100, 250, 500, and 1,000 mg/kg bw. The dose was based on each animal's body weight on Days 0, 7, 14, 21, and 28.

During the study, the animals were evaluated for the effect of the test compound on body weight, food consumption, clinical signs, the eyes, clinical chemistry, and haematology. Gross necropsy evaluations were performed on all adults. Histopathologic evaluation of selected tissues was conducted on the control and high-dose groups.

There were no treatment-related deaths during the study, and no treatment-related effects on body weight or food consumption. There were no treatment-related effects on clinical chemistry values or haematological parameters. There were no treatment-related effects on organ weights, no treatment-related gross or histopathological changes in the organs and tissues.

The NOEL in the 28-day dermal toxicity study in rats was 1,000 mg/kg bw/day based on the absence of toxicity at the highest dose investigated.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 (fluopicolide)
Purity:	97.7%
Batch no.:	2050190//PP241024/2

2. Vehicle and/or positive control

Vehicle:	0.5% aqueous carboxymethylcellulose

3. Test animals

Species:	Rats
Strain:	Wistar Hanover (Crl:WI[Glx/BRL/Han]IGS BR) rats
Age:	8 to 9 weeks old
Weight at start:	232.9-244.6 g for the males, 166.9-171.1 g for the females
Source:	[redacted]
Acclimation period:	Yes
Diet:	PMI Certified Rodent Diet 5002 in "meal" form, St. Louis, MO
Water:	Tap water ad lib
Housing:	Individually housed in suspended stainless steel cages
Temperature:	19-25 °C
Humidity:	30-70%
Air changes:	10-15 cycles/hour
Photoperiod:	12 hours

B. Study design

1. In-life dates: February 24 to March 27, 2003

2. Animal assignment and treatment

The dermal route of exposure was employed in accordance with the test guidelines for a subacute dermal toxicity study. The dose groups for this study were 0 (control group), 100, 250, 500, and 1,000 mg/kg bw, applied dermally. The doses were based on each animal's body weight on Days 0, 7, 14, 21, and 28.

Rats were randomly assigned to dose groups, based on weight, using special computer software.

Table 5.3.3- 1: Study design

Group no.	Dose (mg/kg bw)	Number of males	Number of females
	Treatment phase		
1	0	10	10
2	100	10	10
3	250	10	10
4	500	10	10
5	1,000	10	10

3. Dose preparation and analysis

Three days prior to dosing, the hair was removed from the dorsal and lateral areas of the trunk of each rat using electric clippers. The day of dosing and on subsequent study days, when required due to hair growth, the dose area was clipped with electric clippers.

The test material was weighed out for each animal and applied to a 2 inch by 2 inch two ply gauze pad, backed with a piece of plastic, which had been moistened with 2 mL of 0.5% aqueous carboxymethylcellulose, as water would not moisten the test compound. The gauze was placed on the shaved skin of the rat and secured with porous medical tape (Zonas®, by Johnson and Johnson). The torso of the animal was then wrapped with porous medical tape, to assure that the gauze remained in contact with the skin during the dosing interval. The same procedure was performed for control animals, except for only gauze moistened with approximately 2 mL of 0.5% aqueous carboxymethylcellulose was applied to the dose site.

The test substance/gauze was held in contact with the skin for a minimum of six hours/day for five consecutive days/week for four weeks. During the fifth week, the animals were dosed daily until the day the rat was to be sacrificed (rats were not dosed on the day of sacrifice). Each day the gauze and tape were removed and the dose site was gently wiped with water-dampened and dry paper towels to remove as much test substance residue as feasible without damaging the skin.

4. Statistics

Statistical significance was determined at $p < 0.05$ for all tests, with the exception of Bartlett's test in which a probability value of $p < 0.001$ was used. All tests were two-tailed, except for gross and histopathologic lesion evaluations that were one-tailed.

Continuous data was analysed by Bartlett's test for homogeneity. If the data was homogeneous an ANOVA was performed followed by Dunnett's t-test on parameters showing a significant effect by ANOVA. If the data was non-homogeneous, a Kruskal-Wallis ANOVA was performed followed by the Mann-Whitney U-test to identify statistical significance between groups.

C. Methods

1. Observations

Animals were observed twice daily for morbidity and mortality (except once daily on weekends and holidays). Clinical examinations were conducted once a week.

2. Body weight and food intake

The weight of each animal was determined on Study Days 0, 7, 14, 21, and 28. A terminal body weight was obtained on animals that were found dead or sacrificed in extremis following the day of dosing.

Individual food consumption was determined once a week for all animals.

3. Ophthalmoscopy

Ophthalmic exams were conducted pre-dose and during study week four.

4. Laboratory investigations (clinical chemistry)

During the fourth week of the study, following an overnight fast, blood was collected from the orbital sinus of all rats, under anaesthesia with isoflurane, and was analysed for standard serum chemistry and haematological parameters. During the fifth week of the study, blood was collected from the orbital sinus, under anaesthesia with isoflurane, and was analysed for prothrombin time.

Haematology:

The following parameters were determined:

Table 5.3.3- 2: Haematology parameters

Blood Cell morphologies Erythrocytes (RBC) Haematocrit (HCT) Haemoglobin (Hgb) Leukocytes (WBC) (total & differential)	Mean corpuscular volume (MCV) Mean corpuscular haemoglobin (MCH) Mean corpuscular haemoglobin concentration Platelets (PLTS) Prothrombin time (PT)

Biochemistry:

The following parameters were determined:

Table 5.3.3- 3: Biochemistry parameters

Alanine aminotransferase (ALT)	Gamma-glutamyltranspeptidase (GGT)
Albumin (ALB)	Globulin (Glob)
Alkaline phosphatase (ALP)	Glucose, fasting (Glue)
Aspartate aminotransferase (AST)	Lactic dehydrogenase (LDH)
Bilirubin, total (T-Bili)	Phosphate (Phos)
Blood urea nitrogen (BUN)	Potassium (K)
Calcium (Calc)	Protein, total (T-Prot)
Cholesterol, total (Choi)	Sodium (Na)
Chloride (Cl)	Triglycerides (Trig)
Creatinine (Great)	Uric Acid (Uric-A)
Creatine phosphokinase (CK)	A/G ratio

5. Sacrifice and pathology

Macroscopic examination:

A complete gross examination was performed on all animals that died, were sacrificed moribund, or were sacrificed at study termination. The organs in Table 5.3.3- 4 were weighed. With the exception of the physical identifier (microchip), representative sections of the tissues were preserved in 10% buffered formalin.

Organ weights:

The following organ weights were determined:

Table 5.3.3- 4: Organ weights

Adrenal	Ovaries
Brain	Spleen
Epididymis	Testes
Heart	Thymus
Kidney	Uterus
Liver	

6. Histopathology

Tissue sampling:

The tissues collected from the control (0 mg/kg bw) and high-dose group (1,000 mg/kg bw) animals, which were examined microscopically (see Table 5.3.3- 5), were processed, embedded in paraffin, sectioned, mounted, and stained with haematoxylin and eosin (H&E) for examination under the light microscope. Tissues in which compound-related effects were detected were examined at lower doses, as necessary, to establish no-observed-effect levels.

Table 5.3.3- 5: Tissue sampling

Adrenal	Jejunum	Rectum
Aorta	Joint, fem/tib[a]	Salivary gland
Bone marrow	Kidney	Seminal vesicle
Bone, sternum	Larynx	Skin (treated and untreated)
Brain	Liver	Spinal cord
Cerebrum	Lung	Cervical
Midbrain	Lymph node, cervical	Thoracic
Cerebellum	Lymph node, mesenteric	Lumbar
Medulla	Mammary gland	Spleen
Cecum	Muscle	Stomach, glandular
Cervix[a]	Nasal structure	Stomach, non-glandular
C literal gland[a]	Nasopharynx	Testicle
Colon	Nerve, sciatic (peripheral)	Thymus
Duodenum	Optic nerve	Thyroid/parathyroid
Epididymis	Oral structure	Tongue[a]
Esophagus	Ovary	Tootha
Exoribital/lacrimal gland[a]	Pancreas	Trachea
Eye	Parathyroid	Urinary bladder
Gross lesions	Physical identifier[b]	Uterus
Harderian gland[a]	Pituitary	Vagina[a]
Heart	Preputial gland[a]	Zymbal's gland[a]
Ileum	Prostrate	

[a] Preserved for possible micropathologic evaluation
[b] The identification chip implanted in each animal was collected at necropsy

II. Results and Discussion

A. Results

1. Clinical results

There were no treatment-related clinical signs in any groups.

One animal (female OY 4104) was sacrificed on Study Day 21, due to the skin accidentally being cut when the animal was unwrapped.

2. Body weight and food intake

Body weight

There was no compound-related effect on body weight.

Table 5.3.3- 6: Mean body weight (g)

Day	Dose level (mg/kg bw/day)									
	0	100		250		500		1,000		
Males										
Body weights [g] (*% difference to control*)										
Day 0	244.6	242.9	*(-1)*	243.9	*(±0)*	232.9	*(-5)*	242.2	*(-1)*	
Day 7	263.8	260.5	*(-1)*	261.2	*(-1)*	259.5	*(-2)*	260.5	*(-1)*	
Day 14	282.7	278.1	*(-2)*	280.5	*(-1)*	280.8	*(-1)*	278.5	*(-1)*	
Day 21	302.2	294.4	*(-3)*	297.3	*(-2)*	298.7	*(-1)*	296.3	*(-2)*	
Day 28	311.3	302.2	*(-3)*	301.9	*(-3)*	305.2	*(-2)*	303.7	*(-2)*	
Females										
Body weights [g] (*% difference to control*)										
Day 0	171.1	169.7	*(-1)*	166.9	*(-2)*	167.4	*(-2)*	166.9	*(-2)*	
Day 7	181.0	177.1	*(-2)*	179.9	*(-1)*	182.0	*(+1)*	174.1	*(-4)*	
Day 14	190.6	193.8	*(+2)*	190.9	*(±0)*	199.0	*(+4)*	188.3	*(-1)*	
Day 21	203.5	203.2	*(±0)*	207.1	*(+2)*	211.0	*(+4)*	196.7	*(-3)*	
Day 28	211.5	207.8	*(-2)*	211.8	*(±0)*	217.7	*(+3)*	202.0	*(-4)*	

Food intake

There was no compound-related effect on food consumption.

3. Ophthalmological results

There were no treatment-related findings.

4. Laboratory investigations

Haematology:

There were no compound-related effects on haematology parameters evaluated in this study. There were various parameters for males and prothrombin time for the low-dose group females that were statistically significantly different from the control group, but these findings were not dose related and were not biologically different from the control group.

Clinical chemistry:

There were no compound-related effects on the clinical chemistry parameters evaluated in this study. There were various parameters which were statistically significant from the control group, but these findings were not dose related and/or were not biologically different than the control group.

5. Sacrifice and pathology

Necropsy:

There were no compound-related gross observations. Miscellaneous lesions noted were decreased size of testis/epididymis, discolouration of the thymus, eye lesions, and skin lesions.

Organ weights:

There were no compound-related differences between the absolute and relative organ weights for control and treatment animals.

6. Histopathology

There were no compound-related micropathological lesions in the 1,000 mg/kg dose group. Minimal background changes, typically present in young Wistar rats, were seen in both sexes.

III. Conclusion

The No Observed Effect Level (NOEL) for this study was 1,000 mg/kg bw/day, the highest dose tested.

Assessment and conclusion by applicant:

The study conforms to OECD TG 410 and is valid and acceptable to investigate the short-term dermal toxicity of fluopicolide in rats. A NOAEL of 1000 mg/kg bw/d (the highest dose tested) was determined from this study.

CA 5.4 Genotoxicity testing

Several genotoxicity studies were performed for fluopicolide: six reverse gene mutation tests in *Salmonella typhimurium* and *Escherichia coli* strains of bacteria, one of them recently ([redacted] 2017; M-595228-01-1), one chromosomal aberration assay in Chinese hamster V79 cells *in vitro*, one chromosomal aberration assay in human lymphocytes *in vitro*, one HPRT mutation assay in Chinese hamster V79 cells, two *in vivo* micronucleus assays in mouse bone marrow cells with oral administration, one *in vivo* micronucleus assay in mouse bone marrow cells with intraperitoneal administration, one *in vivo* UDS assay in rat hepatocytes by oral route and one *in vivo* Comet assay in male mice with oral gavage administration.

One of the earlier five bacterial reverse mutation assay showed a very slight increase in the number of revertant colonies in strain (TA 98) only with metabolic activation and to a lesser extent with TA 1537 in the presence of metabolic activation and with the tester strain TA 1535 in the absence of exogenous metabolic activation at the highest concentration of 5000 µg/plate where precipitation was observed. Therefore, this result was considered of doubtful biological significance and four additional assays were conducted to confirm/infirm this equivocal response. No evidence of mutagenic activity of fluopicolide was observed in the four additional bacterial reverse mutation assays performed with five *Salmonella typhimurium* strains and one *Escherichia coli* strain. In addition, a recently conducted bacterial reverse mutation assay ([redacted]; 2017; M-595228-01-1) was also negative and also confirmed the overall negative outcome in this study type. Furthermore, a Comet assay was also recently ([redacted] 2018; M-635020-01-1) performed to confirm the negative profile for the endpoint gene mutation *in vivo*.

The chromosomal aberration assay performed in Chinese hamster V79 showed a positive response. However, the increase of aberrant cells occurred at cytotoxic concentrations where mitotic indices were clearly below the limit of 50% indicating the doubtful biological significance of these data. This chromosome aberration assay was therefore repeated in human lymphocytes and gave a clear negative response. Moreover, two *in vivo* micronucleus assays were performed in mice by the oral route up to the limit dose of 2000 mg/kg bw. Both of these assays were negative. However, one was of questionable biological significance due to the slight increase of micronucleated polychromatic erythrocytes in bone marrow of some animals given 2000 mg/kg bw as well as in one control animal. As the ratio of polychromatic to normochromatic erythrocytes was not significantly affected and no clinical signs were observed in both assays, a third assay was performed in mice by the intraperitoneal route specially to check for bone marrow exposure. This assay gave a clear negative result for clastogenicity *in vivo* at dose levels showing clear cytotoxicity of the bone marrow.

The HPRT mutation assay in Chinese hamster V79 cells was negative. Moreover, the *in vivo* rat hepatocyte UDS assay clearly showed that fluopicolide does not induce damage to DNA.

In summary, 2 out of 15 tests (one *in vitro* bacterial reverse mutation assay and one *in vitro* chromosome aberration assay) gave positive responses of doubtful biological significance. The *in vivo* mutagenicity data from micronucleus tests are sound and it is clear that no mutagenic effects were seen in the tested animals. Clearly, chromosomal damage does not occur *in vivo*. Furthermore, in the carcinogenicity studies (see section CA 10.9) fluopicolide caused an increase in hepatocellular adenomas in male and female mice by a non-genotoxic mechanism considered not relevant to humans, and increased neoplasms were observed only at or above the maximum tolerated dose (MTD). Altogether these findings clearly show that fluopicolide is devoid of any genotoxic potential when tested *in vitro* and *in vivo*. Fluopicolide is therefore not considered to pose a genotoxic hazard for man.

According to the CLP guidance (Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures Version 5.0 July 2017), a genotoxicity classification of fluopicolide is not warranted.

Fluopicolide was discussed at the 53rd meeting of the Committee for Risk Assessment (RAC-53). It was agreed that classification for genotoxicity was not warranted in accordance with the criteria laid out in Regulation (EC) No 1272/2008.

Table 5.4- 1: Overview of genotoxicity studies performed with fluopicolide

Method Guideline GLP compliance Deviations	Test substance Purity Solvent/Vehicle	Relevant study information	Result	Reference
***In vitro* studies**				
Bacterial point mutation assay (Ames test) OECD 471 (1997) GLP	Fluopicolide 97.8% DMSO	Test system: *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, TA 100, and *Escherichia coli* WP2uvrA The following concentrations were tested: Plate incorporation Experiment I: 50; 160; 500; 1600; and 5000 µg/plate (±S9) Experiment II/III (TA98 only): 50; 160; 500; 1600; 2000; 3000; 4000 and 5000 µg/plate (+S9) Pre-incubation Experiment I: 50; 160; 500; 1600; and 5000 µg/plate (±S9)	**Positive** at precipitating dose levels.	[redacted] 2004; M-197239-02-1 KCA 5.4.1/01
Bacterial point mutation assay (Ames test) OECD 471 (1997) GLP	Fluopicolide Purity not reported DMSO	Test system: *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, TA 100, and *Escherichia coli* WP2uvrA The following concentrations were tested: Plate incorporation Experiment I: 1.6; 8; 40; 200; 1000 and 5000 µg/plate (±S9) Experiment II: 31.25; 62.5; 125; 250; 500 and 1000 µg/plate (-S9) Pre-incubation Experiment II: 31.25; 62.5; 125; 250; 500 and 1000 µg/plate (+S9)	**Negative**	[redacted] 2001; M-202931-01-1 KCA 5.4.1/02

Method Guideline GLP compliance Deviations	Test substance Purity Solvent/Vehicle	Relevant study information	Result	Reference
Bacterial point mutation assay (Ames test) OECD 471 (1997) GLP	Fluopicolide 95.6% DMSO	Test system: *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, TA 100, and *Escherichia coli WP2uvrA* The following concentrations were tested: Plate incorporation Experiment I: 1.6; 8; 40; 200; 1000 and 5000 µg/plate (±S9) Experiment II: 31.25; 62.5; 125; 250; 500 and 1000 µg/plate (-S9) Pre-incubation Experiment II: 31.25; 62.5; 125; 250; 500 and 1000 µg/plate (+S9)	**Negative**	[redacted] 2001; M-202927-01-1 KCA 5.4.1/03
Bacterial point mutation assay (Ames test) OECD 471 (1997) GLP	Fluopicolide 95.9% DMSO	Test system: *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, TA 100, and *Escherichia coli WP2uvrA* The following concentrations were tested: Plate incorporation Experiment I: 1.6; 8; 40; 200; 1000 and 5000 µg/plate (-S9) Experiment II: 31.25; 62.5; 125; 250; 500 and 1000 µg/plate (-S9) Pre-incubation Experiment II: 31.25; 62.5; 125; 250; 500 and 1000 µg/plate (+S9)	**Negative**	[redacted] 2001; M-202939-01-1 KCA 5.4.1/04

Method Guideline GLP compliance Deviations	Test substance Purity Solvent/Vehicle	Relevant study information	Result	Reference
Bacterial point mutation assay (Ames test) OECD 471 (1997) GLP	Fluopicolide 99.3% DMSO	Test system: *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, TA 100, and *Escherichia coli WP2uvrA* The following concentrations were tested: Plate incorporation Experiment I: 1.6; 8; 40; 200; 1000 and 5000 µg/plate (±S9) Experiment II: 31.25; 62.5; 125; 250; 500 and 1000 µg/plate (±S9) Pre-incubation Experiment II: 31.25; 62.5; 125; 250; 500 and 1000 µg/plate (+S9)	**Negative**	[redacted] 2001; M-202935-01-1 KCA 5.4.1/05
Bacterial point mutation assay (Ames test) OECD 471 (1997) GLP	Fluopicolide 98.2% DMSO	Test system: *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98 TA 100 and TA 102 The following concentrations were tested: Plate incorporation Experiment I: 3; 10; 33; 100; 333; 1000; 2500; and 5000 µg/plate (±S9) Since for the positive control of strain TA 102 with S9 mix the acceptance criteria were not met, this part of experiment I was repeated (see experiment Ia). Experiment Ia (TA102 only): 3; 10; 33; 100; 333; 1000; 2500; and 5000 µg/plate (+S9) Pre-incubation Experiment II: 10; 33; 100; 333; 1000; 2500 and 5000 µg/plate (±S9)	**Negative**	[redacted] 2017; M-595228-01-1 KCA 5.4.1/09

Method Guideline GLP compliance Deviations	Test substance Purity Solvent/Vehicle	Relevant study information	Result	Reference
In vitro chromosome aberration assay in Chinese hamster lung V79 cells OECD 473 (1997) GLP Cytotoxicity was not measured using the parameters of relative population doubling (RPD) or relative increase in cell count (RICC). Only 25-100 metaphases, instead of the currently required 300 metaphases, were analysed.	Fluopicolide 97.8% DMSO	Test system: Chinese hamster lung V79 cells The following concentrations were tested: Experiment I: 25; 50; 75 and 100 µg/mL (±S9) Experiment II: 1.6; 3.2 and 6.3 µg/mL (-S9)	**Positive** at cytotoxic concentrations	[redacted] 2004; M-197260-02-1 KCA 5.4.1/06
In vitro chromosome aberration assay in human lymphocytes OECD 473 (1997) GLP Cytotoxicity was not measured using the parameters of relative population doubling (RPD) or relative increase in cell count (RICC). Only 25-100 metaphases, instead of the currently required 300 metaphases, were analysed.	Fluopicolide 95.9% DMSO	Test system: Human lymphocytes The following concentrations were tested: Experiment I: 19.53; 78.13 and 156.25 µg/mL (-S9) 78.13; 312.5 and 625 µg/mL (+S9) Experiment II: 1.22; 9.77 and 19.53 µg/mL (-S9) 39.06; 156.25 and 312.5 µg/mL (+S9)	**Negative**	[redacted] 2001; M-201582-01-1 KCA 5.4.1/07

Method Guideline GLP compliance Deviations	Test substance Purity Solvent/Vehicle	Relevant study information	Result	Reference
In vitro HPRT mutation assay in Chinese hamster lung V79 cells OECD 476 (1997) GLP	Fluopicolide 97.8% DMSO	Test system: Chinese hamster lung V79 cells The following concentrations were tested: Experiment I: 1.2; 3.8; 12.1; 38.2; 120.8; 382; 1208 and 3820 µg/mL (±S9) Experiment II: 0.4; 0.8; 1.6; 3.2; 6.3; 12.5; 25; 50; 75; 100 and 120 µg/mL (±S9) Experiment III: 0.313; 0.625; 1.25; 2.5; 5; 10; 20; 30; 40; 50 and 60 µg/mL (±S9)	**Negative**	[redacted] 2005; M-210831-02-1 KCA 5.4.1/08
***In vivo* studies in somatic cells**				
Mouse micronucleus test OECD 474 (1997) GLP Target organ exposure not measured. Only 2000 instead of 4000 erythrocytes were analysed. Only 200 instead of 500 cells were analysed to obtain the PCE/NCE ratio.	Fluopicolide 97.8%M-220782-01-1 2% 1% (w/v) methylcellulose	Test system: Mouse (HsdWin:NMRI) The following concentrations were tested: 200, 600 and 2000 mg/kg bw (oral)	**Negative**	[redacted] 2005; M-197261-02-1 KCA 5.4.2/01
Mouse micronucleus test OECD 474 (1997) GLP Target organ exposure not measured. Only 2000, not 4000 erythrocytes, were analysed. Only 200, not 500 cells were analysed to obtain the PCE/NCE ratio.	Fluopicolide 96.1% 1% (w/v) methylcellulose	Test system: Mouse (Crl:CD1) The following concentration was tested: 2000 mg/kg bw (oral)	**Negative**	[redacted] 2003; M-219364-01-1 KCA 5.4.2/02

Method Guideline GLP compliance Deviations	Test substance Purity Solvent/Vehicle	Relevant study information	Result	Reference
Mouse micronucleus test OECD 474 (1997) GLP Altered NCE/PCE ratio. Only 2000, not 4000 erythrocytes, were analysed.	Fluopicolide 99.4% 0.5% cremophor	Test system: Mouse (Crl:CD1) The following concentrations were tested: 150, 300 and 600 mg/kg bw (i.p.)	**Negative**	[redacted] 2003; M-223119-01-1 KCA 5.4.2/03
Rat UDS assay OECD 486 (1997) GLP	Fluopicolide 97.7% 1% (w/v) methylcellulose	Test system: Rat (Hsd/Ola SD) The following concentrations were tested: 600 and 2000 mg/kg bw (oral)	**Negative**	[redacted] 2000; M-197230-02-1 KCA 5.4.2/04 [redacted] 2000; M-199318-01-1 KCA 5.4.2/05
Comet assay in mice OECD 489 (2016) GLP	Fluopicolide 98.2% 1% (w/v) methylcellulose	Test system: Mouse (Hsd:ICR (CD-1)) The following concentrations were tested: 500, 1000 and 2000 mg/kg bw (oral, gavage)	**Negative**	[redacted] 2018; M-635020-01-1 KCA 5.4.2/06

CA 5.4.1 In vitro studies

Data Point:	KCA 5.4.1/01
Report Author:	[redacted]
Report Year:	2004
Report Title:	Bacterial reverse mutation test - Code: AE C638206 00 1C99 0005
Report No:	C008172
Document No:	M-197259-02-1
Guideline(s) followed in study:	OECD 471 (1997); US-EPA OPPTS 870.5100 (1998); EEC Directive 92/69, B13./14.
Deviations from current test guideline:	Deviations from the current OECD guideline (471, 1997): None.
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

This study was designed to assess the mutagenic potential of fluopicolide in amino acid dependent strains of *Salmonella typhimurium* and a strain of *Escherichia coli*.

Technical fluopicolide was tested for mutagenicity with the strains TA 100, TA 1535, TA 1537 and TA 98 of *Salmonella typhimurium* and with *Escherichia coli* WP2uvrA.

Two independent mutagenicity studies were conducted with all tester strains (one plate incorporation test and one pre-incubation test), each in the absence and in the presence of an Aroclor-induced metabolizing system derived from a rat liver homogenate. To confirm a mutagenic response in the plate incorporation test with the tester strain TA 98 in the presence of S9-mix, two further plate incorporation tests were conducted with this strain using smaller dose intervals.

The test substance was dissolved in DMSO, and each bacterial strain was exposed to 5 to 8 dose levels. Doses ranged from 50 to 5000 µg/plate.

Control plates without mutagen showed that the number of spontaneous revertant colonies was within the laboratory's historical control range and similar to that described in the literature. All positive control compounds showed the expected increase in the number of revertant colonies.

The test substance was not toxic to the bacterial strains in either the presence and in the absence of metabolic activation. Visible precipitation on the plates was observed at 500 µg/plate and above.

In the plate incorporation test fluopicolide gave a dose-dependent increase in the number of revertant colonies with the bacterial strain TA 98. This response was confirmed in the additional tests with this tester strain. In the pre-incubation test an increase in the number of revertant colonies was found with the *Salmonella* strains TA 98 and to a lesser extent with TA 1537 in the presence of metabolic activation and with the tester strain TA 1535 in the absence of exogenous metabolic activation.

Fluopicolide was mutagenic in this bacterial mutation test at precipitating dose levels.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 (fluopicolide), technical
Purity:	97.8% (w/w)
Batch no.:	Mixture of PP/241024/2 & PP/241067/1

2. Vehicle and/or positive control

Vehicle:	DMSO (concentration not given)
Positive control:	Without S9 mix:
	Na-azide: TA 100, TA 1535
	9-aminoacridine: TA 1537
	2-nitrofluorene: TA 98
	4-nitroquinoline-N-oxide: WP2uvrA
	With S9 mix:
	2-Aminoanthracene: TA 98, TA 100, TA 1535, TA 1537, TA 102

3. Activation:

Due to the limited capacity for metabolic activation of potential mutagens in *in vitro* methods, an exogenous metabolic activation system is necessary.

The S9 fraction was prepared by the department conducting the study according to Ames *et al.* (1975)[13]. Male Sprague Dawley rats (200-300 g), supplied by Harlan Winkelmann (33178 Borchen, Germany), received a single intraperitoneal injection of Aroclor 1254 (500 mg/kg bw) 5 days before killing.

The livers were removed from at least 5-6 animals and, using cold sterile solutions at approx. 0 to 4 °C and glassware, were then pooled and washed in approx. 150 mM KCl (approx. 1 mL/g wet liver). The washed livers were cut into small pieces and homogenized in three volumes of KCl. The homogenate was centrifuged at approx. 9000 x g for 10 minutes. The supernatant was the S9 fraction. This was divided into small portions, rapidly frozen and stored at approx. -80 °C for not longer than six months.

The protein content was determined for every batch. Also for every batch of S9 an independent validation was performed with a minimum of two different mutagens, e.g. 2-aminoanthracene and dimethylbenzanthracene, to confirm metabolic activation by microsomal enzymes.

[13] Ames BN, Mccann J, Yamasaki E. Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. Mutat Res. 1975 Dec;31(6):347-64.

S9 Mix:

Sufficient S9 fraction was thawed at room temperature immediately before each test. One volume of S9 fraction (batch no. 99/9 for the first plate incorporation test, protein concentration 56.7 g/L; batch no. 99/10 for the second and third plate incorporation test and for the pre-incubation test, protein concentration 54.3 g/L) was mixed with 9 volumes of the S9 cofactor solution, which was kept on ice until used. This preparation is termed S9-mix. The concentrations of the different compounds in the S9-mix were:

8 mM $MgCl_2$
33 mM KCl
5 mM glucose-6-phosphate
4 mM NADP
100 mM phosphate buffer pH 7.4.

4. Test organisms:

The strains of *Salmonella typhimurium* were obtained from Professor B.N. Ames, University of California, U.S.A. The strain of *Escherichia coli* was obtained from the National Collection of Industrial Bacteria, Aberdeen, Scotland.

Bacteria were grown overnight in nutrient broth (25 g Oxoid Nutrient Broth No. 2 / liter) at approx. 37 °C. The amount of bacteria in the cell suspension was checked by nephelometry. Inoculation was performed with stock cultures which had been stored at approx. -80 °C. The different bacterial strains were checked half-yearly with regard to their respective biotin, histidine and/or tryptophan requirements, membrane permeability, ampicillin resistance, crystal violet sensitivity, UV resistance and response to diagnostic mutagens. All criteria for a valid assay were fulfilled.

Sterility checks and control plates:

Sterility of S9-mix and the test substance were indicated by the absence of contamination on the test substance and S9-mix sterility check plates. Control plates (background control and positive controls) gave the expected number of colonies, i.e. values were within the laboratory's historical control range.

5. Test substance concentrations used:

The test substance was dissolved in DMSO and a stock solution of 50 mg/mL was prepared for the highest concentration, which provided a final concentration of 5000 µg/plate. Further dilutions of 1600, 500,160 and 50 µg/plate were used in the first plate incorporation test.

To verify the findings in the first test with tester strain TA 98 in the presence of S9-mix, two further tests with this strain were conducted. They used smaller intervals between dose levels of 50, 160, 500, 1600, 2000, 3000, 4000 and 5000 µg/plate.

For the second (pre-incubation) test dose levels of 50, 160, 500, 1600 and 5000 µg/plate were chosen for all tester strains and additional concentrations of 2000, 3000 and 4000 µg/plate with the tester strain TA 98.

Visible precipitation of the test substance on the plates was observed at 500 µg/plate and above.

The test substance was not toxic to the bacterial strains.

Based on this, in the plate incorporation and pre-incubation test, the following concentrations were used:

Plate incorporation test: 50, 160, 500, 1600, 5000 µg/plate
Pre-incubation test: 50, 160, 500, 1600, 5000 µg/plate.

B. Test performance

Experimental phase: April 04 to April 28, 2000

1. Assay procedure

Two independent mutation tests were performed unless clearly positive or dose-related activity was observed in the first test. Where results were negative or equivocal, a second test was conducted. This included a pre-incubation step if the first test was clearly negative. Pre-incubation involved incubating the test substance, S9-mix and bacteria for a short period before pouring this mixture onto plates of minimal agar.

Each test was performed in both the presence and absence of S9-mix using all bacterial tester strains and a range of concentrations of the test substance. Positive and negative controls as well as solvent controls were included in each test. Triplicate plates were used.

The highest concentration in the first mutation experiment was 50 mg/mL of the test substance in the chosen solvent, which provided a final concentration of 5000 µg/plate.

Further dilutions of 1600, 500, 160 and 50 µg/plate were used. Dose levels used in the second experiment were based on findings, including toxicity, in the first experiment. A reduction in the number of spontaneously occurring colonies and visible thinning of the bacterial lawn were used as toxicity indicators. Thinning of the bacterial lawn was evaluated microscopically.

In both tests top agar was prepared which, for the *Salmonella* strains, contained 100 mL agar (0.6% (w/v) agar, 0.5% (w/v) NaCl) with 10 mL of a 0.5 mM histidine-biotin solution. For *E. coli* histidine was replaced by tryptophan (2.5 mL, 0.5 mM). The following ingredients were added (in the following order) to 2 mL of molten top agar at approx. 48 °C:

0.5 mL S9-mix (if required) or buffer
0.1 mL of an overnight nutrient broth culture of the bacterial tester strain
0.1 mL test substance solution (dissolved in DMSO)

In the second mutagenicity test if appropriate these top-agar ingredients were pre-incubated by shaking for approx. 20 minutes at approx. 30 °C. After mixing, and pre-incubation if appropriate, the liquid was poured into a petri dish containing a 25 mL layer of minimal agar (1.5% (w/v) agar, Vogel-Bonner E medium with 2% (w/v) glucose). After incubation for approx. 48 hours at approx. 37 °C in the dark, colonies (his* and trp* revertants) were counted by hand or by a suitable automatic colony counter. The counter was calibrated for each test by reading a test pattern plate to verify the manufacturer's requirements for the counter's sensitivity.

2. Statistics

Not given (according to the OECD guideline 471, a statistical analysis of the data is not mandatory).

3. Acceptance / assessment criteria:

The assay is considered valid if the following criteria are met:

- The solvent control data are within the laboratory's normal control range for the spontaneous mutant frequency.
- The positive controls induce increases in the mutation frequency which are significant and within the laboratory's normal range.

4. Evaluation of results, criteria for a positive response

A test substance is classified as mutagenic if it has either of the following effects:

a) It produces at least a 2-fold increase in the mean number of revertants per plate of at least one of the tester strains over the mean number of revertants per plate of the appropriate vehicle control at complete bacterial background lawn

b) It induces a dose-related increase in the mean number of revertants per plate of at least one of the tester strains over the mean number of revertants per plate of the appropriate vehicle control in at least two to three concentrations of the test compound at complete bacterial background lawn.

If the test substance does not achieve either of the above criteria, it is considered to show no evidence of mutagenic activity in this system.

II. Results and Discussion

A. Mutation assays

The test compound was not toxic to the bacterial strains in either the presence or in the absence of metabolic activation. Visible precipitation on the plates was observed at concentrations of ≥ 500 µg/plate.

In the plate incorporation test fluopicolide produced a significant increase (2.8x compared to solvent control) in the number of revertant colonies with the bacterial strain TA 98 with metabolic activation at a precipitating dose level of 5000 µg/plate. A slight increase in mutation frequency (1.4-2.5x compared to the respective solvent controls) was also observed in two further plate incorporation tests with narrower dose spacing with this tester strain at concentrations ≥ 3000 µg/plate with metabolic activation.

In the pre-incubation test an increase in the number of revertant colonies was also found with the *Salmonella* strain TA 98 with metabolic activation at precipitating concentrations ≥ 2000 µg/plate (2.2-2.7x compared to solvent control) and additionally to a lesser extent with TA 1537 in the presence of metabolic activation (2.2x compared to solvent control) and with the tester strain TA 1535 in the absence of exogenous metabolic activation (2.0x compared to solvent control, covered by historical control range) at 5000 µg/plate.

All positive controls produced significant increases in the number of revertant colonies. Thus, the sensitivity of the assay and the efficacy of the exogenous metabolic activation system were demonstrated.

An overview of the results is given in Table 5.4.1-1 to 5.4.1- 3.

Table 5.4.1- 1: Revertant colony counts obtained in the plate incorporation test – Experiment I

Treatment	Concentration (µg/plate)	Mean revertant colony counts in strains				
		TA 98	TA 100	TA 1535	TA 1537	WP2uvrA/ pKM101
Without metabolic activation (-S9)						
Fluopicolide	5000	22.0p	110.0p	10.7p	7.0p	20.0p
	1600	22.3p	138.7p	13.0p	5.3p	19.3p
	500	18.7p	137.7p	7.3p	5.7p	19.0p
	160	26.0	134.0	10	5.0	28.7
	50	24.0	124.7	9.3	4.7	30.7
Historical solvent control mean	-	20.1 ± 4.6	123.4 ± 26.3	8.4 ± 2.5	6.3 ± 2.1	23.8 ± 5.9
Historical control range	-	8.3-40.3	57.7-223.0	3.0-23.3	3.0-14.3	10.7-42.7
Solvent control	-	23.7	116.3	8.0	5.7	27.7
Negative control	-	27.3	163.0	9.7	6.0	26.3
Positive control						
Sodium azide	1	NA	579.7	565.3	NA	NA
4-nitroquinoline-N-oxide	2	NA	NA	NA	NA	447.3
2-nitrofluorene	2.5	342.3	NA	NA	NA	NA
9-Aminoacridine	50	NA	NA	NA	113.3	NA
With metabolic activation (+S9)						
Fluopicolide	5000	69.0p (2.8x)	150.7p	8.3p	10.7p	33.0
	1600	33.0p	118.7p	12.0p	8.3p	29.0
	500	24.0p	128.7p	8.7p	7.3p	30.0
	160	25.0	147.7	8.7	7.0	30.3
	50	25.3	138.0	10.0	8.3	28.7
Historical solvent control mean	-	23.3 ± 5.0	134.4 ± 27.7	8.9 ± 2.1	6.8 ± 2.2	25.0 ± 6.4
Historical control range	-	8.3-38.3	73.0-244.3	4.0-17.7	2.3-14.0	13.0-48.0
Solvent control	-	24.3	139.0	9.3	7.7	34.0
Negative control	-	28.3	154.7	12.0	6.0	30.7
Positive control						
2-aminoanthracene	0.5 - 10 #	1060.0	1284.7	169.3	177.7	194.3

NA: not applicable
#: 0.5 µg/plate for TA 98 and TA 100, 1 µg/plate for strain TA 1535 and TA 1537, and 10 µg/plate for WP2*uvr*A
p: precipitated
Number of experiments for historical control data:
Plate incorporation test: n= 186 (TA 100, ± S9); n= 177 and 179 (TA 1535, +S9 and –S9, respectively); n= 176 and 175 (TA 1537, +S9 and –S9, respectively); n= 179 and 177 (TA 98, +S9 and –S9, respectively); n= 97 (WP2uvrA, ±S9)

Table 5.4.1- 2: Revertant colony counts obtained in the plate incorporation test – confirmation of mutagenic response in strain TA98 with metabolic activation (+S9)

Treatment	Concentration (µg/plate)	Mean revertant colony counts in strain TA 98	
		Experiment II	Experiment III
Fluopicolide	5000	42.0p (1.4x)	74.0p (2.5x)
	4000	60.3p (2.0x)	62.3p (2.1x)
	3000	48.3p (1.6x)	61.3p (2.1x)
	2000	42.3p (1.4x)	41.3p (1.4x)
	1600	37.3p	42.3p (1.4x)
	500	28.3p	29.7p
	160	30.7	24.3
	50	22.7	23.0
Historical solvent control mean	-	23.3 ± 5.0	23.3 ± 5.0
Historical control range	-	8.3-38.3	8.3-38.3
Solvent control	-	30.3	29.3
Negative control	-	26.3	30.0
Positive control 2-aminoanthracene	0.5 - 10 #	712.0	917.7

p: precipitated
#: 0.5 µg/plate for TA 98
Number of experiments for historical control data:
Plate incorporation test: n= 179 (TA 98, +S9)

Table 5.4.1- 3: Revertant colony counts obtained in the pre-incubation test

Treatment	Concentration (µg/plate)	Mean revertant colony counts in strains				
		TA 98	TA 100	TA 1535	TA 1537	WP2uvrA/ pKM101
Without metabolic activation (-S9)						
Fluopicolide	5000	18.0p	90.3p	8.0p (2.0x)	5.3p	27.7p
	1600	22.7p	88.0p	6.3p	5.0p	28.3p
	500	17.7p	80.3p	3.0p	3.7p	29.3p
	160	17.0	81.0	9.7	4.3	27.3
	50	20.0	82.3	5.0	3.7	26.0
Historical solvent control mean	-	20.5 ± 4.6	116.6 ±20.9	7.3 ± 1.6	6.0 ± 1.6	22.0 ± 5.6
Historical control range	-	12.0-32.0	84.3-196.0	3.3-11.0	3.3-12.3	10.0-34.3
Solvent control	-	17.7	91.7	4.0	6.3	22.0
Negative control	-	25.7	114.0	9.7	7.3	24.3
Positive control						
Sodium azide	1	NA	329.0	283.3	NA	NA
4-nitroquinoline-N- oxide	2	NA	NA	NA	NA	179.7
2-nitrofluorene	2.5	465.7	NA	NA	NA	NA
9-Aminoacridine	50	NA	NA	NA	153.0	NA
With metabolic activation (+S9)						
Fluopicolide	5000	70.7p (2.7x)	141.7p	9.7p	12.3p (2.2x)	31.7p
	4000	62.3p (2.3x)	NA	NA	NA	NA
	3000	70.0p (2.6x)	NA	NA	NA	NA
	2000	57.7p (2.2x)	NA	NA	NA	NA
	1600	43.7p	120.3p	8.7p	5.3p	33.7p
	500	31.3p	135.7p	7.0p	7.7p	26.7p
	160	32.3	141.3	9.3	5.0	29.0
	50	25.7	111.7	10.0	4.3	31.3
Historical solvent control mean		24.9 ± 5.0	128.4 ±19.8	8.1 ± 1.6	6.5 ± 1.6	24.6 ± 6.1
Historical control range	-	15.3-40.3	94.7-177.7	4.3-12.0	3.0-11.0	14.3-37.7
Solvent control		26.7	115.7	9.3	5.7	31.0
Negative control	-	30.3	141.0	9.3	4.3	33.0
Positive control 2-aminoanthracene	0.5 - 10 #	829.0	717.0	123.3	120.0	151.0

NA: not applicable
p: precipitated
#: 0.5 µg/plate for TA 98 and TA 100, 1 µg/plate for strain TA 1535 and TA 1537, and 10 µg/plate for WP2*uvr*A

Number of experiments for historical control data:
Pre-incubation test: n= 52 and 61 (TA 100, +S9 and –S9, respectively); n= 65 and 72 (TA 1535, +S9 and –S9, respectively); n= 59 and 71 (TA 1537, +S9 and –S9, respectively); n= 67 and 69 (TA 98, +S9 and –S9, respectively); n= 62 (WP2uvrA, ±S9)

III. Conclusion

Fluopicolide was mutagenic in this bacterial mutation test in TA 98 and to a lesser extent in TA 1537 in the presence of metabolic activation and with the tester strain TA 1535 in the absence of exogenous metabolic activation at precipitating dose levels.

Assessment and conclusion by applicant:

The study was conducted according to OECD TG 471 and is valid and acceptable to assess the mutagenic potential of fluopicolide in *S-typhimurium* and *E. Coli*. Fluopicolide was mutagenic under the conditions of this study (when tested up to precipitative concentrations) in bacterial strains TA 98 and TA 1537 in the presence of S-9 and in strain TA 1535 in the absence of S9.

Data Point:	KCA 5.4.1/02
Report Author:	Ballantyne, M.
Report Year:	2001
Report Title:	Reverse mutation assay in four histidine requiring strains of Salmonella typhimurium and one Trytophan requiring strain of Escherichia coli Code: AE C638206.00 1C99 0005
Report No:	C012562
Document No:	M-202931-01-1
Guideline(s) followed in study:	OECD Guideline 471 (adopted 1997), EEC Annex V Tests B 13 and B 14 (1993), Japanese MHW (1989) and MAFF (1985) and ICH Harmonised Tripartite Guideline (1997) ; US-EPA 870.5100
Deviations from current test guideline:	Deviations from the current OECD guideline (471, 1997): None.
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

Fluopicolide was assayed for mutation in four histidine-requiring strains (TA 98, TA 100, TA 1535 and TA 1537) of *Salmonella typhimurium*, and one tryptophan-requiring strain (WP2 uvrA) of *Escherichia coli*, both in the absence and in the presence of metabolic activation by an Aroclor 1254-induced rat liver post mitochondrial fraction (S9), in two separate experiments.

Experiment I treatments were carried out in all the tester strains using final concentrations of fluopicolide at 1.6 to 5000 µg/plate, plus negative (solvent) and positive controls. Following these treatments, no clear evidence of toxicity was observed, as would normally be indicated by a thinning of the background bacterial lawn. Precipitation of the test substance was observed on all plates treated at 1000 µg/plate and above, indicating that the solubility limit of the test substance within the assay system had been exceeded at the upper end of the dose range.

Experiment II treatments of all tester strains were performed using a maximum test dose of 1000 µg/plate, this being an estimate of the lower limit of the precipitating dose range. A narrowed dose range was employed in order to more closely investigate those concentrations of fluopicolide approaching the maximum test dose, and therefore considered most likely to induce any mutation. In addition, all experiment II treatments performed in the presence of S9 employed a pre-incubation step, in order to increase the range of mutagenic chemicals that could be detected using this assay system. Following experiment II treatments, no evidence of toxicity was observed in any of the test strains.

Precipitation of test agent was observed on all plates treated at 1000 µg/plate, except those of strain WP2 uvrA in the presence of S9.

Negative (solvent) and positive control treatments were included for all strains in both experiments. The mean numbers of revertant colonies on negative control plates were comparable with the acceptable ranges, and were significantly elevated by positive control treatments.

No increases in revertant numbers sufficient to be considered as evidence of any fluopicolide mutagenic activity were observed following any of the test treatments, either in the absence or in the presence of metabolic activation (S9).

It was concluded that fluopicolide did not induce mutation in four strains of *Salmonella typhimurium* (TA 98, TA 100, TA 1535 and TA 1537) and one strain of *Escherichia coli* (WP2 uvrA), when tested under the conditions employed for this study. These conditions included treatments at concentrations up to precipitating test doses, both in the absence and in the presence of a rat liver metabolic activation system (S9).

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 00 IC99 0005 (fluopicolide)
Purity:	Not given in report
Batch no.:	OP2050190

2. Vehicle and/or positive control

Vehicle:	DMSO (concentration not given)
Positive control	Without S9 mix:
	Na-azide: TA 100, TA 1535
	9-aminoacridine: TA 1537
	2-nitrofluorene: TA 98
	4-nitroquinoline-N-oxide: WP2uvrA
	With S9 mix:
	Benzo[a]pyrene: TA 98
	2-aminoanthracene: TA 100, TA 1535, TA 1537, TA 102, WP2uvrA

3. Activation:

Due to the limited capacity for metabolic activation of potential mutagens in in vitro methods an exogenous metabolic activation system is necessary.

The mammalian liver post-mitochondrial fraction (S9) used for metabolic activation was prepared from male Sprague Dawley rats induced with Aroclor 1254 and obtained from Molecular Toxicology Incorporated, USA. Batches of MolToxTM S9 were stored frozen at -80 °C, and thawed just prior to incorporation into the top agar. Each batch was checked by the manufacturer for sterility, protein content, ability to convert ethidium bromide and cyclophosphamide to bacterial mutagens, and cytochrome P-450-catalysed enzyme activities (alkoxyresorufin-0-dealkylase activities).

A quality control statement, relating to the batch of S9 preparation used, was included in the report.

4. Test organisms:

Four strains of *Salmonella typhimurium* (TA 98, TA 100, TA 1535 and TA 1537) and one strain of *Escherichia coli* (WP2 uvrA) were used in this study. The *Salmonella typhimurium* tester strains were originally obtained from the UK NCTC, and the *Escherichia coli* strain from the Cancer Research Unit, University of York. For all assays, bacteria were cultured for 10 hours at 37±1 °C in nutrient broth (containing ampicillin for strains TA 98 and TA 100). Incubation was carried out in a shaking incubator. Bacteria were taken from vials of frozen cultures, which had been checked for strain characteristics of histidine or tryptophan dependence, rfa character (*Salmonella* strains), uvrB character (*Salmonella* strains), uvrA character (*Escherichia coli*) and presence (strains TA 98 and TA 100) or absence (strains TA 1535, TA 1537 and *Escherichia coli*) of the pKM101 ampicillin resistance factor.

Checks were carried out according to Maron and Ames[14] and De Serres and Shelby[15]. All experimentation commenced within 2 hours of the end of the period of incubation.

5. Test substance concentrations used:

Fluopicolide, batch number OP2050190, was a beige powder, which was received on 21 July 2000. It was stored at room temperature in the dark. Purity and certificate of analysis was not supplied from the sponsor. The expiry date of the test substance was stated to be July 2002. Determinations of the stability and characteristics of the test substance were the responsibility of the Sponsor.

Test substance solutions were prepared by dissolving fluopicolide in sterile anhydrous analytical grade dimethyl sulphoxide (DMSO), with the aid of vortexing in experiment I, immediately prior to assay to give the maximum required treatment solution concentration. This solution was filter-sterilized (Gelman Acrodisc CR filter, 0.2 µm pore size) and further dilutions were made using DMSO. The test substance solutions were protected from light and used within approx. 5 hours of the initial formulation of the test substance. Solutions were used as follows:

Experiment I (+/- S9): 1.6, 8, 40, 200, 1000, 5000 µg/plate
Experiment II (+/- S9): 31.25, 62.6, 125, 250, 500, 1000 µg/plate

[14] Maron D M and Ames B N (1983) Revised methods for the Salmonella mutagenicity test. Mutation Research 113, 173-215.5

[15] De Serres F J and Shelby M D (1979) Recommendations on data production and analysis using the Salmonella/microsome mutagenicity assay. Mutation Research 64, 159-165.

B. Test performance

Experimental phase: July 25 to August 21, 2000

1. Assay procedure

Fluopicolide was tested in four strains of *Salmonella typhimurium* (TA 98, TA 100, TA 1535 and TA 1537) and one strain of *Escherichia coli* (WP2 uvrA), in two separate experiments, at the concentrations detailed previously, using triplicate plates without and with S9. Negative (solvent) controls were included in each assay, in quintuplicate without and with S9. In each experiment, bacterial strains were treated with diagnostic mutagens in triplicate in the absence of S9. The activity of the S9 mix used in each experiment was confirmed by AAQ or B[a]P treatments (again in triplicate) of the strains in the presence of S9. Platings were achieved by the following sequence of additions to 2.5 mL molten agar at 46±1 °C:

- 0.1 mL bacterial culture
- 0.1 mL test article solution or control
- 0.5 mL 10 % S9 mix or buffer solution

followed by rapid mixing and pouring on to Vogel-Bonner E agar plates. When set, the plates were inverted and incubated at 37±1 °C in the dark for 3 days. Following incubation, these plates were examined for evidence of toxicity to the background lawn, and where possible revertant colonies were counted (see Colony counting).

As the results of the first experiment were negative, treatments in the presence of S9 in experiment II included a pre-incubation step. Quantities of test article or control solution (reduced to 0.05 mL), bacteria and S9 mix detailed above, were mixed together and incubated for 1 hour at 37±1 °C, with shaking, before the addition of 2.5 mL molten agar at 46±1 °C. Plating of these treatments then proceeded as for the normal plate-incorporation procedure. In this way, it was hoped to increase the range of mutagenic chemicals that could be detected in the assay.

Volume additions for the experiment II pre-incubation treatments were reduced to 0.05 mL due to the solvent (DMSO) employed in this study. This and some other organic solvents are known to be near to toxic levels when added at volumes of 0.1 mL in this assay system when employing the pre-incubation methodology. By reducing the addition volume to 0.05 mL per plate, it was hoped to minimise or eliminate any toxic effects of the solvent that may have otherwise occurred.

Colonies were counted electronically using a Seescan Colony Counter (Seescan plc) or manually where confounding factors such as split agar or the presence of precipitate affected the accuracy of the automated counter. The background lawn was inspected for signs of toxicity.

Individual plate counts from both experiments were recorded separately and the mean and standard deviation of the plate counts for each treatment were determined.

The accepted normal ranges for mean numbers of spontaneous revertants on solvent control plates for this laboratory are consistent with ranges of spontaneous revertants per plate considered acceptable elsewhere[15]. The accepted normal ranges for mean numbers of induced revertants on positive control plates for this laboratory are based on a large volume of historical control data accumulated from experiments where the correct strain and assay functioning were considered to have been confirmed.

2. Statistics

The m-statistic was calculated to check that the data were Poisson-distributed and Dunnett's test was used to compare the counts of each dose with the control. The presence or otherwise of a dose response was checked by linear regression analysis.

3. Acceptance / assessment criteria:

The assay was considered valid if the following criteria were met:

1. The mean negative control counts fell within the normal ranges of the laboratory.
2. The positive control chemicals induced clear increases in revertant numbers confirming discrimination between different strains, and an active S9 preparation.
3. No more than 5 % of the plates were lost through contamination or some other unforeseen event.

4. Evaluation of results, criteria for a positive response

A test substance is classified as mutagenic if it has either of the following effects:

1. The assay was valid (see point 3 above).
2. Dunnett's test gave a significant response ($p \leq 0.01$) and the data set(s) showed a significant dose correlation.
3. The positive responses described above were reproducible.

II. Results and Discussion

A. Mutation assays

Experiment I treatments were carried out in all the tester strains using final concentrations of fluopicolide at 1.6 to 5000 µg/plate, plus negative (solvent) and positive controls. Following these treatments, no clear evidence of toxicity was observed, as would normally be indicated by a thinning of the background bacterial lawn and/or a marked reduction in revertant numbers. Precipitation of the test article was observed on all plates treated at 1000 µg/plate and above, indicating that an appropriate dose limiting effect had occurred, as the solubility limit of the test article within the assay system had been exceeded at the upper end of the dose range.

Experiment II treatments of all the tester strains were performed using a maximum test dose of 1000 µg/plate, this being an estimate of the lower limit of the precipitating dose range. A narrowed dose range was employed in order to more closely investigate those concentrations of fluopicolide approaching the maximum test dose, and therefore considered most likely to induce any mutation. In addition, all experiment II treatments in the presence of S9 included a pre-incubation step. Following all treatments in experiment II, no evidence of toxicity was observed in any of the test strains. Precipitation of test agent was recorded during the treatment of all plates at the maximum test dose of 1000 µg/plate, but after incubation of the test plates observations of precipitation at the time of revertant colony scoring excluded strain WP2 uvrA plates treated in the presence of S9. Due to the observations made at the time of plate treatment, and those in experiment I, the maximum test dose of these treatments was considered to have been very close to a precipitating dose level.

The individual plate counts were averaged to give mean values. From the data it can be seen that mean solvent control counts were comparable with the normal historical ranges as shown in the following tables. The positive control chemicals all induced large increases in revertant numbers in the appropriate strains, which fell within or above the normal historical ranges. Less than 5 % of plates were lost, leaving adequate numbers of plates at all treatments. The study therefore demonstrated correct strain and assay functioning and was accepted as valid.

The mutation data were evaluated as follows. Fluopicolide treatments of all the test strains resulted in only a single increase in revertant numbers that was statistically significant when the data were analysed at the 1% level using Dunnett's test. This increase occurred following experiment II treatments of strain TA1535 in the absence of S9, but failed to demonstrate any clear dose-relationship, occurring at an intermediate dose only. Furthermore, the data within this study indicated that this increase was not reproducible, as no similar increases were observed following comparable strain treatments in experiment I. The observed increase in revertant numbers was therefore attributed to chance, and this study was considered to have provided no clear evidence of any fluopicolide mutagenic activity.

An overview of the results is given in Table 5.4.1- 4 and Table 5.4.1- 5.

Table 5.4.1- 4: Mean revertant colony counts/plate in experiment I

Treatment	Concentration (µg/plate)	Mean revertant colony counts in strains				
		TA 98	TA 100	TA 1535	TA 1537	WP2uvrA/ pKM101
Without metabolic activation (-S9)						
Fluopicolide	5000	52p	109p	28p	17p	18p
	1000	45p	102p	31p	12p	19p
	200	50	108	26	18	19
	40	38	105	30	16	13
	8	55	103	28	22	15
	1.6	42	114	29	15	21
Historical solvent control mean	-	37.9 ± 9.5	98.1 ± 16.9	15.5 ± 4.1	10.5 ± 6.3	12.0 ± 4.2
Historical control range	-	14 – 62	55 – 143	5 – 26	1 – 34	1 – 23
Solvent control	-	44	114	24	14	20
Positive control						
Sodium azide	2	NA	626	573	NA	NA
4-nitroquinoline-N-oxide	5	NA	NA	NA	NA	657
2-nitrofluorene	5	1107	NA	NA	NA	NA
Benzo[a]pyrene	10	390	NA	NA	NA	NA
9-Aminoacridine	50	NA	NA	NA	224	NA
With metabolic activation (+S9)						
Fluopicolide	5000	65p	107p	17p	26p	25p
	1000	50p	114p	26p	14p	15p
	200	51	102	20	20	20
	40	42	97	21	23	25
	8	52	113	18	21	20
	1.6	49	111	20	18	23
Historical solvent control mean	-	38.2 ± 9.6	104.8 ±22.4	18.9 ± 5.2	17.1 ± 7.0	16.8 ± 5.8
Historical control range	-	14 – 63	47 – 163	5 – 32	1 – 35	2 – 32
Solvent control	-	51	98	21	18	25
Positive control 2-aminoanthracene	5 - 10 #	NA	1757	187	266	242

NA: not applicable
p: precipitation
#: 5 µg/plate for TA 100, TA 1535 and TA 1537 and 10 µg/plate for WP2*uvr*A
Historical solvent control data for *S.typhimurium* strains and the *E.coli* strain are derived from at least 17 and 16 data sets, respectively, from the period of 1998-1999.

Table 5.4.1- 5: Mean revertant colony counts/plate in experiment II

Treatment	Concentration (µg/plate)	Mean revertant colony counts in strains				
		TA 98	TA 100	TA 1535	TA 1537	WP2uvrA/ pKM101
Without metabolic activation (-S9)						
Fluopicolide	1000	42p	127p	21p	13p	16p
	500	38	136	24	14	19
	250	36	145	35	14	16
	125	40	143	43**	14	13
	62.5	34	144	30	16	15
	31.25	36	138	29	13	9
Solvent control	-	35 ± 15	122 ± 11	28 ± 2	14 ± 2	16 ± 2
Positive control						
Sodium azide	2	NA	757	735	NA	NA
4-nitroquinoline-N-oxide	2	NA	NA	NA	NA	925
2-nitrofluorene	5	1444	NA	NA	NA	NA
Benzo[a]pyrene	10	300	NA	NA	NA	NA
9-Aminoacridine	50	NA	NA	NA	242	NA
With metabolic activation (+S9) – Pre-incubation test						
Fluopicolide	1000	45p	128p	27p	21p	33
	500	44	116	23	15	35
	250	40	122	23	17	23
	125	34	122	19	13	21
	62.5	40	126	22	18	17
	31.25	41	130	25	18	21
Solvent control	-	39	128	23	15	23
Positive control						
2-aminoanthracene	5 - 10 #	NA	1871	220	428	38

**: p ≤ 0.01, statistically significant compared to control (Dunnett´s test)
NA: not applicable
p: precipitated
#: 0.5 µg/plate for TA 98 and TA 100, 1 µg/plate for strain TA 1535 and TA 1537, and 10 µg/plate for WP2*uvr*A

III. Conclusion

It was concluded that fluopicolide was not genotoxic in the bacterial gene mutation assay under the conditions of this assay. It did not induce mutation in four strains of *Salmonella typhimurium* (TA 98, TA 100, TA 1535 and TA 1537) and one strain of *Escherichia coli* (WP2 uvrA), when tested under the conditions employed for this study. These conditions included treatments at concentrations up to precipitating test doses, both in the absence and in the presence of a rat liver metabolic activation system (S9).

Assessment and conclusion by applicant:

The study was conducted according to OECD TG 471 and is valid and acceptable to assess the mutagenic potential of fluopicolide in *S-typhimurium* and *E. Coli*. Fluopicolide is not mutagenic in bacterial cells under the conditions of this study when tested up to precipitating concentrations (+/- S9).

Data Point:	KCA 5.4.1/03
Report Author:	[redacted]
Report Year:	2001
Report Title:	Reverse mutation assay in four histidine requiring strains of Salmonella typhimurium and one Trytophan requiring strain of Escherichia coli Code: AE C638206 00 1C96 0002
Report No:	C012560
Document No:	M-202927-01-1
Guideline(s) followed in study:	OECD 471 (1997); EEC Annex V Tests B 13 and B 14 (1993); JMHW (1988); JMAFF (1985); ICH Harmonised Tripartite Guideline (1997)
Deviations from current test guideline:	Deviations from the current OECD guideline (471, 1997): None.
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

Fluopicolide was assayed for mutation in four histidine-requiring strains (TA 98, TA 100, TA 1535 and TA 1537) of *Salmonella typhimurium*, and one tryptophan-requiring strain (WP2 uvrA) of *Escherichia coli*, both in the absence and in the presence of metabolic activation by an Aroclor 1254-induced rat liver post-mitochondrial fraction (S9), in two separate experiments.

Experiment I treatments were carried out in all tester strains using final concentrations of fluopicolide at 1.6 to 5000 µg/plate, plus negative (solvent) and positive controls. Following these treatments, no clear evidence of toxicity was observed, as would normally be indicated by a thinning of the background bacterial lawn. Precipitation of the test substance was observed on all plates treated at 1000 µg/plate and above, indicating that the solubility limit of the test substance within the assay system had been exceeded at the upper end of the dose range.

Experiment II treatments of all the tester strains were performed using a maximum test dose of 1000 µg/plate, this being an estimate of the lower limit of the precipitating dose range. A narrowed dose range was employed in order to more closely investigate those concentrations of fluopicolide approaching the maximum test dose, and therefore considered most likely to induce any mutation. In addition, all experiment II treatments performed in the presence of S9 employed a pre-incubation step, in order to increase the range of mutagenic chemicals that could be detected using this assay system. Following experiment II treatments, no evidence of toxicity was observed in any of the test strains. Precipitation of test agent was observed on all plates treated at the maximum test dose of 1000 µg/plate.

Negative (solvent) and positive control treatments were included for all strains in both experiments. The mean numbers of revertant colonies on negative control plates were comparable with the acceptable ranges and were significantly elevated by positive control treatments.

No increases in revertant numbers sufficient to be considered as evidence of any fluopicolide mutagenic activity were observed following any of the test treatments, either in the absence or in the presence of metabolic activation (S9).

It was concluded that fluopicolide did not induce mutation in four strains of *Salmonella typhimurium* (TA 98, TA 100, TA 1535 and TA 1537) and one strain of *Escherichia coli* (WP2 uvrA), when tested under the conditions employed for this study. These conditions included treatments at concentrations up to precipitating test doses, both in the absence and in the presence of a rat liver metabolic activation system (S9).

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 00 1C96 0002 (OP 2050045) (fluopicolide)
Purity:	95.6%
Batch no.:	OP2050045

2. Vehicle and/or positive control

Vehicle:	Sterile anhydrous analytical grade dimethyl sulphoxide (DMSO) (concentration not given)
Positive control:	Without S9 mix: Na-azide: TA 100, TA 1535 9-aminoacridine: TA 1537 2-nitrofluorene: TA 98 4-nitroquinoline-N-oxide: WP2uvrA With S9 mix: Benzo[a]pyrene: TA 98 2-aminoanthracene: TA 100, TA 1535, TA 1537, TA 102, WP2uvrA

3. Activation:

Due to the limited capacity for metabolic activation of potential mutagens in *in vitro* methods an exogenous metabolic activation system is necessary.

The mammalian liver post-mitochondrial fraction (S9) used for metabolic activation was prepared from male Sprague Dawley rats induced with Aroclor 1254 and obtained from Molecular Toxicology Incorporated, USA. Batches of MolToxTM S9 were stored frozen at -80 °C, and thawed just prior to incorporation into the top agar. Each batch was checked by the manufacturer for sterility, protein content, ability to convert ethidium bromide and cyclophosphamide to bacterial mutagens, and cytochrome P-450-catalysed enzyme activities (alkoxyresorufin-O-dealkylase activities).

A quality control statement, relating to the batch of S9 preparation used, was included in the report.

4. Test organisms:

Four strains of *Salmonella typhimurium* (TA 98, TA 100, TA 1535 and TA 1537) and one strain of *Escherichia coli* (WP2 uvrA) were used in this study. The *Salmonella typhimurium* tester strains were originally obtained from the UK NCTC, and the *Escherichia coli* strain from the Cancer Research Unit, University of York. For all assays, bacteria were cultured for 10 hours at 37±1 °C in nutrient broth (containing ampicillin for strains TA 98 and TA 100). Incubation was carried out in a shaking incubator. Bacteria were taken from vials of frozen cultures, which had been checked for strain characteristics of histidine or tryptophan dependence, rfa character (*Salmonella* strains), uvrB character (*Salmonella* strains), uvrA character (*Escherichia coli*) and presence (strains TA 98 and TA 100) or absence (strains TA 1535, TA 1537 and *Escherichia coli*) of the pKM101 ampicillin resistance factor.

Checks were carried out according to Maron and Ames[14] and De Serres and Shelby[15]. All experimentation commenced within 2 hours of the end of the period of incubation.

5. Test substance concentrations used:

Fluopicolide, batch number OP2050045, was a fine beige powder, which was received on 7 August 2000. It was stored at room temperature in the dark. Purity was stated as 95.6%. Test substance solutions were prepared by dissolving fluopicolide in sterile anhydrous analytical grade dimethyl sulphoxide (DMSO), immediately prior to assay to give the maximum required treatment solution concentration. This solution was filter-sterilized and further dilutions were made using DMSO. The test substance solutions were protected from light and used within approximately 5½ hours of the initial formulation of the test substance. Solutions were used as follows:

Experiment I (+/- S9): 1.6, 8, 40, 200, 1000, 5000 µg/plate
Experiment II (+/- S9): 31.25, 62.6, 125, 250, 500, 1000 µg/plate

B. Test performance

Experimental phase: August 10 to August 22, 2000

1. Assay procedure

Fluopicolide was tested in four strains of *Salmonella typhimurium* (TA 98, TA 100, TA 1535 and TA 1537) and one strain of *Escherichia coli* (WP2 uvrA), in two separate experiments, at the concentrations detailed previously, using triplicate plates without and with S9. Negative (solvent) controls were included in each assay, in quintuplicate without and with S9. In each experiment, bacterial strains were treated with diagnostic mutagens in triplicate in the absence of S9. The activity of the S9 mix used in each experiment was confirmed by AAN or B[a]P treatments (again in triplicate) of the strains in the presence of S9. Platings were achieved by the following sequence of additions to 2.5 mL molten agar at 46±1 °C:

- 0.1 mL bacterial culture
- 0.1 mL test article solution or control
- 0.5 mL 10 % S9 mix or buffer solution

followed by rapid mixing and pouring on to agar plates. When set, the plates were inverted and incubated at 37±1 °C, in the dark for 3 days. Following incubation, these plates were examined for evidence of toxicity to the background lawn, and where possible revertant colonies were counted (see Colony counting).

As the results of the first experiment were negative, treatments in the presence of S9 in experiment II included a pre-incubation step. Quantities of test article or control solution (reduced to 0.05 mL), bacteria and S9 mix detailed above, were mixed together and incubated for 1 hour at 37±1 °C, with shaking, before the addition of 2.5 mL molten agar at 46 ± 1 °C. Plating of these treatments then proceeded as for the normal plate-incorporation procedure. In this way, it was hoped to increase the range of mutagenic chemicals that could be detected in the assay.

Volume additions for the experiment II pre-incubation treatments were reduced to 0.05 mL due to the solvent (DMSO) employed in this study. This and some other organic solvents are known to be near to toxic levels when added at volumes of 0.1 mL in this assay system when employing the pre-incubation methodology. By reducing the addition volume to 0.05 mL per plate, it was hoped to minimise or eliminate any toxic effects of the solvent that may have otherwise occurred.

Colonies were counted electronically using a Seescan Colony Counter or manually where confounding factors such as split agar or the presence of precipitate affected the accuracy of the automated counter. The background lawn was inspected for signs of toxicity.

Individual plate counts from both experiments were recorded separately and the mean and standard deviation of the plate counts for each treatment were determined.

The accepted normal ranges for mean numbers of spontaneous revertants on solvent control plates for this laboratory are consistent with ranges of spontaneous revertants per plate considered acceptable elsewhere[15]. The accepted normal ranges for mean numbers of induced revertants on positive control plates for this laboratory are based on a large volume of historical control data accumulated from experiments where the correct strain and assay functioning were considered to have been confirmed.

2. Statistics

The m-statistic was calculated to check that the data were Poisson-distributed and Dunnett's test was used to compare the counts of each dose with the control. The presence or otherwise of a dose response was checked by linear regression analysis.

3. Acceptance / assessment criteria:

The assay was considered valid if the following criteria were met:

1. The mean negative control counts fell within the normal ranges of the laboratory.
2. The positive control chemicals induced clear increases in revertant numbers, confirming discrimination between different strains, and an active S9 preparation.
3. No more than 5 % of the plates were lost through contamination or some other unforeseen event.

4. Evaluation of results, criteria for a positive response

A test substance is classified as mutagenic if it has either of the following effects:

1. The assay was valid (see point 3 above).
2. Dunnett's test gave a significant response ($p < 0.01$) and the data set(s) showed a significant dose correlation.
3. The positive responses described above were reproducible.

II. Results and Discussion

A. Mutation assays

Experiment I treatments were carried out in all the tester strains using final concentrations of fluopicolide at 1.6 to 5000 µg/plate, plus negative (solvent) and positive controls. Following these treatments, no clear evidence of toxicity was observed, as would normally be indicated by a thinning of the background bacterial lawn and/or a marked reduction in revertant numbers.

Precipitation of the test substance was observed on all plates treated at 1000 µg/plate and above, indicating that an appropriate dose limiting effect had occurred, as the solubility limit of the test substance within the assay system had been exceeded at the upper end of the dose range.

Experiment II treatments of all the tester strains were performed using a maximum test dose of 1000 ug/plate, this being an estimate of the lower limit of the precipitating dose range. A narrowed dose range was employed in order to more closely investigate those concentrations of fluopicolide approaching the maximum test dose, and therefore considered most likely to induce any mutation. In addition, all experiment II treatments in the presence of S9 included a pre-incubation step. Following all the treatments in experiment II, no evidence of toxicity was observed in any of the test strains. Precipitation of test agent was observed on all plates treated at 1000 µg/plate.

The individual plate counts were averaged to give mean values, which are presented in the following tables. From the data it can be seen that mean solvent control counts were comparable with the normal historical ranges.

The positive control chemicals all induced large increases in revertant numbers in the appropriate strains, which fell within or above the normal historical ranges. Less than 5 % of plates were lost, leaving adequate numbers of plates at all treatments. The study therefore demonstrated correct strain and assay functioning and was accepted as valid.

No fluopicolide treatments of any of the test strains, either in the absence or in the presence of S9, resulted in any increases in revertant numbers that were statistically significant when the data were analysed at the 1% level using Dunnett's test. This study was therefore considered to have provided no evidence of any fluopicolide mutagenic activity.

An overview of the results is given in Table 5.4.1- 6 and Table 5.4.1- 7.

Table 5.4.1- 6: Summary of the incidence of revertant colony counts obtained per plate in experiment I

Treatment	Concentration (µg/plate)	Mean revertant colony counts in strains				
		TA 98	TA 100	TA 1535	TA 1537	WP2uvrA/pKM101
Without metabolic activation (-S9)						
Fluopicolide	5000	27p	89p	20p	9p	21p
	1000	39p	97p	25p	19p	25p
	200	35	92	30	16	21
	40	31	116	34	20	24
	8	38	101	24	15	22
	1.6	37	108	28	16	25
Historical solvent control mean		37.9 ± 9.5	98.1 ± 16.9	15.5 ± 4.1	12.5 ± 6.3	12.0 ± 4.2
Historical control range	-	14 – 62	55 – 142	5 – 26	1 – 34	1 – 23
Solvent control		34	112	26	18	23
Positive control						
Sodium azide	2	NA	654	613	NA	NA
4-nitroquinoline-N-oxide	5	NA	NA	NA	NA	616
2-nitrofluorene	5	1032	NA	NA	NA	NA
Benzo[a]pyrene	10	260	NA	NA	NA	NA
9-Aminoacridine	50	NA	NA	NA	159	NA

Treatment	Concentration (µg/plate)	Mean revertant colony counts in strains				
		TA 98	TA 100	TA 1535	TA 1537	WP2uvrA/ pKM101
With metabolic activation (+S9)						
Fluopicolide	5000	48p	108p	23p	13p	23p
	1000	44p	135p	28p	12p	29p
	200	37	134	27	18	24
	40	37	131	26	13	24
	8	47	135	21	18	24
	1.6	39	136	19	13	19
Historical solvent control mean	-	38.2 ± 9.6	104.8 ±22.4	18.9 ± 5.2	17.1 ± 7.0	16.8 ± 5.8
Historical control range	-	14 – 63	47 – 163	5 – 32	1 – 35	2 – 32
Solvent control	-	39	128	25	21	24
Positive control 2-aminoanthracene	5 - 10 #	NA	2652	278	198	293

NA: not applicable
p: precipitated
#: 5 µg/plate for TA 100, TA 1535 and TA 1537, and 10 µg/plate for WP2*uvr*A
Historical solvent control data for *S.typhimurium* strains and the *E.coli* strain are derived from at least 17 and 16 data sets, respectively, from the period of 1998-1999.

Table 5.4.1- 7: Summary of the revertant colony counts obtained per plate in experiment II

Treatment	Concentration (µg/plate)	Mean revertant colony counts in strains				
		TA 98	TA 100	TA 1535	TA 1537	WP2uvrA/ pKM101
Without metabolic activation (-S9)						
Fluopicolide	1000	36p	151p	15p	10p	14p
	500	44	143	22	10	18
	250	41	140	25	13	19
	125	37	144	21	13	15
	62.5	33	151	22	14	16
	31.25	30	146	21	17	16
Solvent control		31	138	25	16	17
Positive control						
Sodium azide	2	NA	836	548	NA	NA
4-nitroquinoline-N-oxide	2	NA	NA	NA	NA	522
2-nitrofluorene	5	1445	NA	NA	NA	NA
Benzo[a]pyrene	10	301	NA	NA	NA	NA
9-Aminoacridine	50	NA	NA	NA	188	NA

Treatment	Concentration (µg/plate)	Mean revertant colony counts in strains				
		TA 98	TA 100	TA 1535	TA 1537	WP2uvrA/ pKM101
With metabolic activation (+S9) – Pre-incubation test						
Fluopicolide	1000	32p	121p	25p	20p	36p
	500	25	111	24	15	29
	250	35	123	22	13	27
	125	35	124	23	15	20
	62.5	39	112	23	16	19
	31.25	30	112	33	12	23
Solvent control	-	32	116	23	14	25
Positive control 2-aminoanthracene	5 - 10 #	NA	1764	200	394	41

NA: not applicable
p: precipitated
#: 0.5 µg/plate for TA 98 and TA 100, 1 µg/plate for strain TA 1535 and TA 1537, and 10 µg/plate for WP2*uvr*A

III. Conclusion

It was concluded that fluopicolide did not induce mutation in four strains of *Salmonella typhimurium* (TA 98, TA 100, TA 1535 and TA 1537) and one strain of *Escherichia coli* (WP2 uvrA), when tested under the conditions employed for this study. These conditions included treatments at concentrations up to precipitating test doses, both in the absence and in the presence of a rat liver metabolic activation system (S9).

Assessment and conclusion by applicant:

The study was conducted according to OECD TG 471 and is valid and acceptable to assess the mutagenic potential of fluopicolide in *S.typhimurium* and *E. Coli*. Fluopicolide is not mutagenic in bacterial cells under the conditions of this study when tested up to precipitating concentrations (+/- S9).

Data Point:	KCA 5.4.1/04
Report Author:	[redacted]
Report Year:	2001
Report Title:	AE C638206 00 1C96 0001 (OP2050046): Reverse mutation assay in four histidine requiring strains of Salmonella typhimurium and one trytophan-requiring strain of Escherichia coli
Report No:	C012566
Document No:	M-202939-01-1
Guideline(s) followed in study:	ICH (1997); JMAFF (1985); JMHW (1989); OECD 471 (1997); Annex V Test B13, B14 (1993)
Deviations from current test guideline:	Deviations from the current OECD guideline (471, 1997): None.
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

Fluopicolide was assayed for mutation in four histidine-requiring strains (TA 98, TA 100, TA 1535 and TA 1537) of *Salmonella typhimurium*, and one tryptophan-requiring strain (WP2 uvrA) of *Escherichia coli*, both in the absence and in the presence of metabolic activation by an Aroclor 1254-induced rat liver post-mitochondrial fraction (S9), in two separate experiments.

Experiment I treatments were carried out in all the tester strains using final concentrations of fluopicolide at 1.6 to 5000 µg/plate, plus negative (solvent) and positive controls. Following these treatments, no clear evidence of toxicity was observed, as would normally be indicated by a thinning of the background bacterial lawn. Some reductions in revertant numbers at the highest test dose in some tester strains may have been the result of test article toxicity.

Precipitation of the test substance was observed on all plates treated at 1000 µg/plate and above, indicating that the solubility limit of the test substance within the assay system had been exceeded at the upper end of the dose range.

Experiment II treatments of all the tester strains were performed using a maximum test dose of 1000 µg/plate, this being an estimate of the lower limit of the precipitating dose range. A narrowed dose range was employed in order to more closely investigate those concentrations of fluopicolide approaching the maximum test dose, and therefore considered most likely to induce any mutation. In addition, all experiment II treatments performed in the presence of S9 employed a pre-incubation step, in order to increase the range of mutagenic chemicals that could be detected using this assay system. Following experiment II treatments, clear evidence of toxicity was only observed at the maximum test dose in strain TA 98 in the absence of S9. Precipitation of test agent was observed on all plates treated at concentrations of 500 µg/plate and above.

Negative (solvent) and positive control treatments were included for all strains in both experiments. The mean numbers of revertant colonies on negative control plates were comparable with the acceptable ranges and were significantly elevated by positive control treatments.

No increases in revertant numbers sufficient to be considered as evidence of any fluopicolide mutagenic activity were observed following any of the test treatments, either in the absence or in the presence of metabolic activation (S9).

It was concluded that fluopicolide did not induce mutation in four strains of *Salmonella typhimurium* (TA 98, TA 100, TA 1535 and TA 1537) and one strain of *Escherichia coli* (WP2 uvrA), when tested under the conditions employed for this study. These conditions included treatments at concentrations up to precipitating test doses, both in the absence and in the presence of a rat liver metabolic activation system (S9).

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 00 1C96 0001 (OP 2050046) (fluopicolide)
Purity:	95.9%
Batch no.:	OP2050046

2. Vehicle and/or positive control

Vehicle:	Sterile anhydrous analytical grade dimethyl sulphoxide (DMSO) (concentration not given)
Positive control:	Without S9 mix: Na-azide: TA 100, TA 1535 9-aminoacridine: TA 1537 2-nitrofluorene: TA 98 4-nitroquinoline-N-oxide: WP2uvrA With S9 mix: Benzo[a]pyrene : TA 98 2-aminoanthracene: TA 100, TA 1535, TA 1537, TA 102, WP2uvrA

3. Activation:

Due to the limited capacity for metabolic activation of potential mutagens in *in vitro* methods an exogenous metabolic activation system is necessary.

The mammalian liver post-mitochondrial fraction (S9) used for metabolic activation was prepared from male Sprague Dawley rats induced with Aroclor 1254 and obtained from Molecular Toxicology Incorporated, USA. Batches of MolToxTM S9 were stored frozen at -80 °C, and thawed just prior to incorporation into the top agar. Each batch was checked by the manufacturer for sterility, protein content, ability to convert ethidium bromide and cyclophosphamide to bacterial mutagens, and cytochrome P-450-catalysed enzyme activities (alkoxyresorufin-0-dealkylase activities).

A quality control statement, relating to the batch of S9 preparation used, was included in the report.

4. Test organisms:

Four strains of *Salmonella typhimurium* (TA 98, TA 100, TA 1535 and TA 1537) and one strain of *Escherichia coli* (WP2 uvrA) were used in this study. The *Salmonella typhimurium* tester strains were originally obtained from the UK NCTC, and the *Escherichia coli* strain from the Cancer Research Unit, University of York. For all assays, bacteria were cultured for 10 hours at 37±1 °C in nutrient broth (containing ampicillin for strains TA 98 and TA 100). Incubation was carried out in a shaking incubator. Bacteria were taken from vials of frozen cultures, which had been checked for strain characteristics of histidine or tryptophan dependence, rfa character (*Salmonella* strains), uvrB character (*Salmonella* strains), uvrA character (*Escherichia coli*) and presence (strains TA 98 and TA 100) or absence (strains TA 1535, TA 1537 and *Escherichia coli*) of the pKM101 ampicillin resistance factor.

Checks were carried out according to Maron and Ames[14] and De Serres and Shelby[15]. All experimentation commenced within 2 hours of the end of the period of incubation.

5. Test substance concentrations used:

Fluopicolide, batch number OP2050046, is a fine beige powder, which was received on 7 August 2000. It was stored at room temperature in the dark. Purity was stated as 95.9% w. The expiry date of the test substance was stated to be 12 October 2000 according to the Sponsor's certificates of analysis. Determinations of the stability and characteristics of the test substance were the responsibility of the Sponsor.

Test substance solutions were prepared by dissolving fluopicolide in sterile anhydrous analytical grade dimethyl sulphoxide (DMSO), immediately prior to assay to give the maximum required treatment solution concentration. This solution was filter-sterilized and further dilutions were made using DMSO. The test substance solutions were protected from light and used within approximately 5½ hours of the initial formulation of the test substance. Solutions were used as follows:

Experiment I (+/- S9): 1.6, 8, 40, 200, 1000, 5000 µg/plate
Experiment II (+/- S9): 31.25, 62.6, 125, 250, 500, 1000 µg/plate

B. Test performance

Experimental phase: July 25 to August 22, 2000

1. Assay procedure

Fluopicolide was tested in four strains of *Salmonella typhimurium* (TA 98, TA 100, TA 1535 and TA 1537) and one strain of *Escherichia coli* (WP2 uvrA), in two separate experiments, at the concentrations detailed previously, using triplicate plates without and with S9. Negative (solvent) controls were included in each assay, in quintuplicate without and with S9. In each experiment, bacterial strains were treated with diagnostic mutagens in triplicate in the absence of S9. The activity of the S9 mix used in each experiment was confirmed by AAN or B[a]P treatments (again in triplicate) of the strains in the presence of S9.

Platings were achieved by the following sequence of additions to 2.5 mL molten agar at 46 ± 1 °C:

- 0.1 mL bacterial culture
- 0.1 mL test article solution or control
- 0.5 mL 10% S9 mix or buffer solution

followed by rapid mixing and pouring on to agar plates. When set, the plates were inverted and incubated at 37 ± 1 °C in the dark for 3 days. Following incubation, these plates were examined for evidence of toxicity to the background lawn, and where possible revertant colonies were counted (see Colony counting).

Initial experiment I treatments were performed using a supply of test substance that due to inconsistencies with labelling and associated paper work, had some doubt over whether the sample was the correct and intended test substance. These data were therefore invalidated and are not presented in this report. A further supply of test substance was therefore obtained and the experiment I treatments repeated in order to provide the valid data that are presented in this report.

As the results of the first experiment were negative, treatments in the presence of S9 in experiment II included a pre-incubation step. Quantities of test substance or control solution (reduced to 0.05 mL), bacteria and S9 mix detailed above, were mixed together and incubated for 1 hour at 37 ± 1 °C, with shaking, before the addition of 2.5 mL molten agar at 46 ± 1 °C. Plating of these treatments then proceeded as for the normal plate-incorporation procedure. In this way, it was hoped to increase the range of mutagenic chemicals that could be detected in the assay.

Volume additions for the experiment II pre-incubation treatments were reduced to 0.05 mL due to the solvent (DMSO) employed in this study. This, and some other organic solvents, are known to be near to toxic levels when added at volumes of 0.1 mL in this assay system when employing the pre-incubation methodology. By reducing the addition volume to 0.05 mL per plate, it was hoped to minimise or eliminate any toxic effects of the solvent that may have otherwise occurred.

Colonies were counted electronically using a Seescan Colony Counter (Seescan plc) or manually where confounding factors such as split agar or the presence of excessive precipitate affected the accuracy of the automated counter. The background lawn of each plate was inspected for signs of toxicity.

Individual plate counts from both experiments were recorded separately and the mean and standard deviation of the plate counts for each treatment were determined.

The accepted normal ranges for mean numbers of spontaneous revertants on solvent control plates for this laboratory are consistent with ranges of spontaneous revertants per plate considered acceptable elsewhere[15]. The accepted normal ranges for mean numbers of induced revertants on positive control plates for this laboratory are based on a large volume of historical control data accumulated from experiments where the correct strain and assay functioning were considered to have been confirmed.

2. Statistics

The m-statistic was calculated to check that the data were Poisson-distributed and Dunnett's test was used to compare the counts of each dose with the control. The presence or otherwise of a dose response was checked by linear regression analysis.

3. Acceptance / assessment criteria:

The assay was considered valid if the following criteria were met:

1. The mean negative control counts fell within the normal ranges of the laboratory.
2. The positive control chemicals induced clear increases in revertant numbers confirming discrimination between different strains, and an active S9 preparation.
3. No more than 5% of the plates were lost through contamination or some other unforeseen event.

4. Evaluation of results, criteria for a positive response

A test substance is classified as mutagenic if it has either of the following effects:

1. The assay was valid (see point 3 above)
2. Dunnett's test gave a significant response ($p < 0.01$) and the data set(s) showed a significant dose correlation
3. The positive responses described above were reproducible.

II. Results and Discussion

A. Mutation assays

Experiment I treatments were carried out in all the tester strains using final concentrations of fluopicolide at 1.6 to 5000 µg/plate, plus negative (solvent) and positive controls. Following these treatments, no clear evidence of toxicity was observed, as would normally be indicated by a thinning of the background bacterial lawn. However, some small reductions in revertant numbers were observed at the maximum test dose treatments with several test strains, and these may have been due to test substance toxicity. Precipitation of the test substance was observed on all plates treated at 1000 µg/plate and above, indicating that an appropriate dose-limiting effect had occurred, as the solubility limit of the test substance within the assay system had been exceeded at the upper end of the dose range.

Experiment II treatments of all the tester strains were performed using a maximum test dose of 1000 ug/plate, this being an estimate of the lower limit of the precipitating dose range. A narrowed dose range was employed in order to more closely investigate those concentrations of fluopicolide approaching the maximum test dose, and therefore considered most likely to induce any mutation. In addition, all experiment II treatments in the presence of S9 included a pre-incubation step.

Following all the treatments in experiment II, no evidence of toxicity was observed in any of the test strains with the exception of strain TA 98 treatments in the absence of S9. Here a slight thinning of the background bacterial lawn was observed at the maximum test dose only. Precipitation of test substance was again observed, on this occasion on all plates treated at 500 µg/plate and above.

The individual plate counts were averaged to give mean values. From the following tables it can be seen that mean solvent control counts were comparable with the normal historical ranges.

The positive control chemicals all induced large increases in revertant numbers in the appropriate strains, which fell within or above the normal historical ranges. Less than 5 % of plates were lost, leaving adequate numbers of plates at all treatments. The study therefore demonstrated correct strain and assay functioning and was accepted as valid.

The mutation data were evaluated as follows. Following fluopicolide treatments of all the test strains, both in the absence and in the presence of a rat liver metabolic activation system (S9), only experiment II treatments of strain TA 100 in the absence of S9 (see Table 5.4.1- 9) provided a statistically significant increase in revertant numbers when the data were analysed at the 1% level using Dunnett's test. This increase was very small in magnitude, failed to show a clear dose-relationship, occurring at an intermediate test dose, and also failed to demonstrate any reproducibility within this study, as comparable experiment I treatments failed to provide any similar increases in revertant numbers. The observed increase was therefore attributed to chance, and this study was considered to have provided no clear evidence of any fluopicolide mutagenic activity.

An overview of the results is given in Table 5.4.1- 8 and Table 5.4.1- 9.

Table 5.4.1- 8: Revertant colony counts obtained per plate in experiment I

Treatment	Concentration (µg/plate)	Mean revertant colony counts in strains				
		TA 98	TA 100	TA 1535	TA 1537	WP2*uvr*A/ pKM101
Without metabolic activation (-S9)						
Fluopicolide	5000	28p	94p	16p	14p	18p
	1000	34p	100p	24p	14p	21p
	200	33	117	30	11	24
	40	32	100	33	16	19
	8	31	107	22	9	20
	1.6	33	108	28	17	16
Historical solvent control mean	-	37.9 ± 9.5	98.1 ± 16.9	15.5 ± 4.1	17.5 ± 6.3	12.0 ± 4.2
Historical control range	-	14 – 62	55 – 142	5 – 26	3 – 34	1 – 23
Solvent control	-	33	114	25	24	24
Positive control						
Sodium azide	2	NA	660	618	NA	NA
4-nitroquinoline-N-oxide	2	NA	NA	NA	NA	641
2-nitrofluorene	5	1041	NA	NA	NA	NA
Benzo[a]pyrene	10	261	NA	NA	NA	NA
9-Aminoacridine	50	NA	NA	NA	152	NA
With metabolic activation (+S9)						
Fluopicolide	5000	43p	115p	17p	22p	19p
	1000	33p	116p	21p	17p	29p
	200	29	114	22	12	27
	40	31	130	22	14	28
	8	32	123	25	10	27
	1.6	38	129	28	21	26
Historical solvent control mean	-	38.2 ± 9.6	104.8±22.4	18.9 ± 5.2	17.1 ± 7.0	16.8 ± 5.8
Historical control range	-	14 – 63	47 – 163	5 – 32	1 – 35	2 – 32
Solvent control	-	42	128	25	23	24
Positive control						
2-aminoanthracene	5 - 10#	NA	2532	280	396	305

NA: not applicable
p: precipitated
#: 5 µg/plate for TA 100, TA 1535 and TA 1537 and 10 µg/plate for WP2*uvr*A
Historical solvent control data for *S.typhimurium* strains and the *E.coli* strain are derived from at least 17 and 16 data sets, respectively, from the period of 1998-1999.

Table 5.4.1- 9: Revertant colony counts obtained per plate in experiment II

Treatment	Concentration (µg/plate)	Mean revertant colony counts in strains				
		TA 98	TA 100	TA 1535	TA 1537	WP2*uvr*A/ pKM101
Without metabolic activation (-S9)						
Fluopicolide	1000	33p	151p	22p	10p	15p
	500	26p	149p	26p	16p	19p
	250	28	156**	23	12	17
	125	35	131	30	15	19
	62.5	34	134	30	18	19
	31.25	30	127	27	9	17
Solvent control	-	31	128	28	13	15
Positive control						
Sodium azide	2	NA	826	790	NA	NA
4-nitroquinoline- N-oxide	2	NA	NA	NA	NA	997
2-nitrofluorene	5	1520	NA	NA	NA	NA
Benzo[a]pyrene	10	325	NA	NA	NA	NA
9-Aminoacridine	50	NA	NA	NA	283	NA
With metabolic activation (+S9) – Pre-incubation test						
Fluopicolide	1000	41p	142p	25p	15p	24p
	500	41p	132p	22p	17p	28p
	250	34	143	17	13	18
	125	37	129	22	12	20
	62.5	43	127	24	14	21
	31.25	37	139	18	17	20
Solvent control	-	35	128	21	16	21
Positive control						
2-aminoanthracene	5 - 10 #	NA	2435	254	459	51

**: $p \leq 0.01$, statistically significant compared to control (Dunnett´s test)
NA: not applicable
p: precipitated
#: 0.5 µg/plate for TA 98 and TA 100, 1 µg/plate for strain TA 1535 and TA 1537, and 10 µg/plate for WP2*uvr*A

III. Conclusion

It was concluded that fluopicolide did not induce mutation in four strains of *Salmonella typhimurium* (TA 98, TA 100, TA 1535 and TA 1537) and one strain of *Escherichia coli* (WP2 uvrA), when tested under the conditions employed for this study. These conditions included treatments at concentrations up to precipitating test doses, both in the absence and in the presence of a rat liver metabolic activation system (S9).

Assessment and conclusion by applicant:

The study was conducted according to OECD TG 471 and is valid and acceptable to assess the mutagenic potential of fluopicolide in *S-typhimurium* and *E. Coli*. Fluopicolide is not mutagenic in bacterial cells under the conditions of this study when tested up to precipitating concentrations (+/- S9).

Data Point:	KCA 5.4.1/05
Report Author:	[redacted]
Report Year:	2001
Report Title:	Reverse mutation assay in four histidine requiring strains of Salmonella typhimurium and one Trytophan requiring strain of Escherichia coli Code: AE C638206 00 1B99 0002
Report No:	C012564
Document No:	M-202935-01-1
Guideline(s) followed in study:	OECD 471 (1997); EEC Annex V Tests B 13, B 14 (1993); JMHW (1988); JMAFF (1985); ICH Harmonised Tripartite Guideline (1997); US-EPA 870.5100
Deviations from current test guideline:	Deviations from the current OECD guideline (471, 1997): None.
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

Fluopicolide was assayed for mutation in four histidine-requiring strains (TA 98, TA 100, TA 1535 and TA 1537) of *Salmonella typhimurium*, and one tryptophan-requiring strain (WP2 uvrA) of *Escherichia coli*, both in the absence and in the presence of metabolic activation by an Aroclor 1254-induced rat liver post-mitochondrial fraction (S9), in two separate experiments.

Experiment I treatments were carried out in all the tester strains using final concentrations of fluopicolide at 1.6 to 5000 µg/plate, plus negative (solvent) and positive controls. Following these treatments, no evidence of toxicity was observed, as would normally be indicated by a thinning of the background bacterial lawn. Precipitation of the test substance was observed on all plates treated at 1000 µg/plate and above, indicating that the solubility limit of the test substance within the assay system had been exceeded at the upper end of the dose range.

Experiment II treatments of all the tester strains were performed using a maximum test dose of 1000 ug/plate, this being an estimate of the lower limit of the precipitating dose range. A narrowed dose range was employed in order to more closely investigate those concentrations of fluopicolide approaching the maximum test dose, and therefore considered most likely to induce any mutation. In addition, all experiment II treatments performed in the presence of S9 employed a pre-incubation step, in order to increase the range of mutagenic chemicals that could be detected using this assay system. Following experiment II treatments, clear evidence of toxicity was only observed at the maximum test dose in strain TA 98 in the absence of S9. Precipitation of test substance was observed on all plates treated at concentrations of 500 µg/plate and above.

Negative (solvent) and positive control treatments were included for all strains in both experiments. The mean numbers of revertant colonies on negative control plates were comparable with the acceptable ranges, and were significantly elevated by positive control treatments.

No increases in revertant numbers sufficient to be considered as evidence of any fluopicolide mutagenic activity were observed following any of the test treatments, either in the absence or in the presence of metabolic activation (S9).

It was concluded that fluopicolide did not induce mutation in four strains of *Salmonella typhimurium* (TA 98, TA 100, TA 1535 and TA 1537) and one strain of *Escherichia coli* (WP2 uvrA), when tested under the conditions employed for this study. These conditions included treatments at concentrations up to precipitating test doses, both in the absence and in the presence of a rat liver metabolic activation system (S9).

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 00 1B99 0002 (R001737) (fluopicolide)
Purity:	99.3%
Batch no.:	R001737

2. Vehicle and/or positive control

Vehicle:	Sterile anhydrous analytical grade dimethyl sulphoxide (DMSO) (concentration not given)
Positive control:	Without S9 mix: Na-azide: TA 100, TA 1535 9-aminoacridine: TA 1537 2-nitrofluorene: TA 98 4-nitroquinoline-N-oxide: WP2uvrA With S9 mix: Benzo[a]pyrene: TA 98 2-aminoanthracene: TA 100, TA 1535, TA 1537, TA 102, WP2uvrA

3. Activation:

Due to the limited capacity for metabolic activation of potential mutagens in *in vitro* methods an exogenous metabolic activation system is necessary.

The mammalian liver post-mitochondrial fraction (S9) used for metabolic activation was prepared from male Sprague Dawley rats induced with Aroclor 1254 and obtained from Molecular Toxicology Incorporated, USA. Batches of MolToxTM S9 were stored frozen at -80 °C, and thawed just prior to incorporation into the top agar. Each batch was checked by the manufacturer for sterility, protein content, ability to convert ethidium bromide and cyclophosphamide to bacterial mutagens, and cytochrome P-450-catalysed enzyme activities (alkoxyresorufin-O-dealkylase activities).

A quality control statement, relating to the batch of S9 preparation used, was included in the report.

4. Test organisms:

Four strains of *Salmonella typhimurium* (TA 98, TA 100, TA 1535 and TA 1537) and one strain of *Escherichia coli* (WP2 uvrA) were used in this study. The *Salmonella typhimurium* tester strains were originally obtained from the UK NCTC, and the *Escherichia coli* strain from the Cancer Research Unit, University of York. For all assays, bacteria were cultured for 10 hours at 37±1 °C in nutrient broth (containing ampicillin for strains TA 98 and TA 100). Incubation was carried out in a shaking incubator. Bacteria were taken from vials of frozen cultures, which had been checked for strain characteristics of histidine or tryptophan dependence, rfa character (*Salmonella* strains), uvrB character (*Salmonella* strains), uvrA character (*Escherichia coli*) and presence (strains TA 98 and TA 100) or absence (strains TA 1535, TA 1537 and *Escherichia coli*) of the pKM101 ampicillin resistance factor.

Checks were carried out according to Maron and Ames[14] and De Serres and Shelby[15]. All experimentation commenced within 2 hours of the end of the period of incubation.

5. Test substance concentrations used:

Fluopicolide, batch number R001737, was a beige powder, which was received on 10 July 2000. It was stored at room temperature in the dark. Purity was stated as 99.3%. The expiry date of the test substance was stated to be 8 December 2000 according to the Sponsor's certificate of analysis.

Test substance solutions were prepared by dissolving fluopicolide in sterile anhydrous analytical grade dimethyl sulphoxide (DMSO), with warming at 37 °C and vortexing in experiment I, immediately prior to assay to give the maximum required treatment solution concentration. This solution was filter-sterilised (Gelman Acrodisc CR filter, 0.2 µm pore size) and further dilutions were made using DMSO. The test article solutions were protected from light and used within approx. 3½ hours of the initial formulation of the test substance.

Solutions were used as follows:

Experiment I (+/- S9): 1.6, 8, 40, 200, 1000, 5000 µg/plate
Experiment II (+/- S9): 31.25, 62.6, 125, 250, 500, 1000 µg/plate

B. Test performance

Experimental phase: July 25 to August 22, 2000

1. Assay procedure

Fluopicolide was tested in four strains of *Salmonella typhimurium* (TA 98, TA 100, TA 1535 and TA 1537) and one strain of *Escherichia coli* (WP2 uvrA), in two separate experiments, at the concentrations detailed previously, using triplicate plates without and with S9. Negative (solvent) controls were included in each assay, in quintuplicate without and with S9. In each experiment, bacterial strains were treated with diagnostic mutagens in triplicate in the absence of S9. The activity of the S9 mix used in each experiment was confirmed by AAN or B[a]P treatments (again in triplicate) of the strains in the presence of S9.

Platings were achieved by the following sequence of additions to 2.5 mL molten agar at46±l °C:

• 0.1 mL bacterial culture
• 0.1 mL test substance solution or control
• 0.5 mL 10% S9 mix or buffer solution

followed by rapid mixing and pouring on to agar plates. When set, the plates were inverted and incubated at 37±1 °C in the dark for 3 days. Following incubation, these plates were examined for evidence of toxicity to the background lawn and where possible revertant colonies were counted (see Colony counting).

As the results of the first experiment were negative, treatments in the presence of S9 in experiment II included a pre-incubation step. Quantities of test article or control solution (reduced to 0.05 mL), bacteria and S9 mix detailed above, were mixed together and incubated for 1 hour at 37±1 °C, with shaking, before the addition of 2.5 mL molten agar at 46±1 °C. Plating of these treatments then proceeded as for the normal plate incorporation procedure. In this way, it was hoped to increase the range of mutagenic chemicals that could be detected in the assay. Volume additions for the experiment II pre-incubation treatments were reduced to 0.05 mL due to the solvent (DMSO) employed in this study. This, and some other organic solvents, are known to be near to toxic levels when added at volumes of 0.1 mL in this assay system when employing the pre-incubation methodology. By reducing the addition volume to 0.05 mL per plate, it was hoped to minimise or eliminate any toxic effects of the solvent that may have otherwise occurred.

Colonies were counted electronically using a Seescan Colony Counter (Seescan plc) or manually where confounding factors such as split agar or the presence of excessive precipitate affected the accuracy of the automated counter. The background lawn of each plate was inspected for signs of toxicity.

Individual plate counts from both experiments were recorded separately and the mean and standard deviation of the plate counts for each treatment were determined.

The accepted normal ranges for mean numbers of spontaneous revertants on solvent control plates for this laboratory are consistent with ranges of spontaneous revertants per plate considered acceptable elsewhere[15]. The accepted normal ranges for mean numbers of induced revertants on positive control plates for this laboratory are based on a large volume of historical control data accumulated from experiments where the correct strain and assay functioning were considered to have been confirmed.

2. Statistics

The m-statistic was calculated to check that the data were Poisson-distributed and Dunnett's test was used to compare the counts of each dose with the control. The presence or otherwise of a dose response was checked by linear regression analysis.

3. Acceptance / assessment criteria:

The assay was considered valid if the following criteria were met:

1. The mean negative control counts fell within the normal ranges of the laboratory.
2. The positive control chemicals induced clear increases in revertant numbers confirming discrimination between different strains, and an active S9 preparation.
3. No more than 5 % of the plates were lost through contamination or some other unforeseen event.

4. Evaluation of results, criteria for a positive response

A test substance is classified as mutagenic if it has either of the following effects:

1. The assay was valid (see point 3 above).
2. Dunnett's test gave a significant response ($p < 0.01$) and the data set(s) showed a significant dose correlation.
3. The positive responses described above were reproducible.

II. Results and Discussion

A. Mutation assays

Experiment I treatments were carried out in all the tester strains using final concentrations of fluopicolide at 1.6 to 5000 µg/plate, plus negative (solvent) and positive controls. Following these treatments, no evidence of toxicity was observed, as would normally be indicated by a thinning of the background bacterial lawn and/or a marked reduction in revertant numbers.

Precipitation of the test substance was observed on all plates treated at 1000 µg/plate and above, indicating that an appropriate dose limiting effect had occurred, as the solubility limit of the test substance within the assay system had been exceeded at the upper end of the dose range.

Experiment II treatments of all the tester strains were performed using a maximum test dose of 1000 ng/plate, this being an estimate of the lower limit of the precipitating dose range. A narrowed dose range was employed in order to more closely investigate those concentrations of fluopicolide approaching the maximum test dose, and therefore considered most likely to induce any mutation. In addition, all experiment II treatments in the presence of S9 included a pre-incubation step.

Following all the treatments in experiment II, no evidence of toxicity was observed in any of the test strains, with the exception of strain TA 98 treatments in the absence of S9. Here a slight thinning of the background bacterial lawn was observed at the maximum test dose only. Precipitation of test substance was again observed, on this occasion on all plates treated at 500 µg/plate and above.

The individual plate counts were averaged to give mean values. From the following tables it can be seen that mean solvent control counts fell within the normal historical ranges. The positive control chemicals all induced large increases in revertant numbers in the appropriate strains. Less than 5% of plates were lost, leaving adequate numbers of plates at all treatments. The study therefore demonstrated correct strain and assay functioning and was accepted as valid.

The evaluation of the mutation data revealed that no fluopicolide treatments of any of the test strains, either in the absence or in the presence of S9, resulted in any increases in revertant numbers that were statistically significant when the data were analysed at the 1% level using Dunnett's test.

This study was therefore considered to have provided no evidence of any fluopicolide mutagenic activity.

An overview of the results is given in Table 5.4.1- 10 and Table 5.4.1- 11.

Table 5.4.1- 10: Revertant colony counts obtained per plate in experiment I

Treatment	Concentration (µg/plate)	Mean revertant colony counts in strains				
		TA 98	TA 100	TA 1535	TA 1537	WP2uvrA/ pKM101
Without metabolic activation (-S9)						
Fluopicolide	5000	42p	91p	20p	14p	13p
	1000	50p	103p	34p	17p	25p
	200	48	95	22	14	18
	40	48	97	26	19	21
	8	49	108	26	21	17
	1.6	50	105	22	16	20
Historical solvent control mean	-	37.9 ± 9.5	98.1 ± 16.9	15.5 ± 4.1	17.5 ± 6.3	12.0 ± 4.2
Historical control range		14 – 62	55 – 142	5 – 26	1 – 34	1 - 23
Solvent control	-	44	109	23	14	20
Positive control						
Sodium azide	2	NA	622	574	NA	NA
4-nitroquinoline-N-oxide	2	NA	NA	NA	NA	645
2-nitrofluorene	5	1051	NA	NA	NA	NA
Benzo[a]pyrene	10	296	NA	NA	NA	NA
9-Aminoacridine	50	NA	NA	NA	227	NA

Treatment	Concentration (µg/plate)	Mean revertant colony counts in strains				
		TA 98	TA 100	TA 1535	TA 1537	WP2uvrA/ pKM101
With metabolic activation (+S9)						
Fluopicolide	5000	37p	108p	21p	19p	17p
	1000	50p	118p	22p	18p	19p
	200	49	111	25	19	19
	40	50	104	18	17	18
	8	56	110	20	21	22
	1.6	60	114	22	20	20
Historical solvent control mean	-	38.2 ± 9.6	104.8±22.4	18.9 ± 5.2	17.1 ± 7.0	16.8 ± 5.8
Historical control range	-	14 – 63	47 – 163	5 – 32	1 – 35	2 – 32
Solvent control	-	50	99	20	18	23
Positive control						
2-aminoanthracene	5 - 10 #	NA	1764	184	266	241

NA: not applicable
p: precipitated
#: 5 µg/plate for TA 100, TA 1535 and TA 1537 and 10 µg/plate for WP2uvrA
Historical solvent control data for *S.typhimurium* strains and the *E.coli* strain are derived from at least 17 and 16 data sets, respectively, from the period of 1998-1999.

Table 5.4.1- 11: Revertant colony counts obtained per plate in experiment II

Treatment	Concentration (µg/plate)	Mean revertant colony counts in strains				
		TA 98	TA 100	TA 1535	TA 1537	WP2uvrA/ pKM101
Without metabolic activation (-S9)						
Fluopicolide	1000	26p	130p	25p	14p	10p
	500	24p	131p	26p	8p	14p
	250	26	129	21	18	16
	125	28	133	24	13	12
	62.5	29	149	25	12	15
	31.25	30	129	26	9	12
Solvent control	-	31	128	29	15	16
Positive control						
Sodium azide	2	NA	654	700	NA	NA
4-nitroquinoline-N-oxide	2	NA	NA	NA	NA	854
2-nitrofluorene	5	1362	NA	NA	NA	NA
Benzo[a]pyrene	10	330	NA	NA	NA	NA
9-Aminoacridine	50	NA	NA	NA	240	NA

Treatment	Concentration (µg/plate)	Mean revertant colony counts in strains				
		TA 98	TA 100	TA 1535	TA 1537	WP2uvrA/ pKM101
With metabolic activation (+S9) – Pre-incubation test						
Fluopicolide	1000	32p	133p	18p	15p	17p
	500	32p	123p	21p	15p	22p
	250	42	141	22	20	21
	125	33	127	25	17	20
	62.5	39	133	21	15	17
	31.25	36	131	24	12	12
Solvent control	-	33	129	21	14	22
Positive control 2-aminoanthracene	5 - 10 #	NA	2074	246	444	49

NA: not applicable
p: precipitated
#: 0.5 µg/plate for TA 98 and TA 100, 1 µg/plate for strain TA 1535 and TA 1537, and 10 µg/plate for WP2*uvr*A

III. Conclusion

It was concluded that fluopicolide did not induce mutation in four strains of *Salmonella typhimurium* (TA 98, TA 100, TA 1535 and TA 1537) and one strain of *Escherichia coli* (WP2 uvrA), when tested under the conditions employed for this study. These conditions included treatments at concentrations up to precipitating test doses, both in the absence and in the presence of a rat liver metabolic activation system (S9).

Assessment and conclusion by applicant:
The study was conducted according to OECD TG 471 and is valid and acceptable to assess the mutagenic potential of fluopicolide in *S-typhimurium* and *E. Coli*. Fluopicolide is not mutagenic in bacterial cells under the conditions of this study when tested up to precipitating concentrations (+/- S9).

Data Point:	KCA 5.4.1/09
Report Author:	[redacted]
Report Year:	2017
Report Title:	Fluopicolide, technical: Salmonella typhimurium reverse mutation assay
Report No:	1835400
Document No:	M-595228-01-1
Guideline(s) followed in study:	OECD 471 (1997); Commission Regulation (EC) No. 440/2008 B13/14 (2008); US-EPA 712-C-98-247, OPPTS 870.5100 (1998)
Deviations from current test guideline:	Deviations from the current OECD guideline (471, 1997): None.
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

In this study the potential of technical fluopicolide to induce gene mutations according to the plate incorporation test (experiment I and Ia) and the pre-incubation test (experiment II) using the *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, TA 100, and TA 102 was investigated.

The assay was performed in three independent experiments both with and without liver microsomal activation. Each concentration, including the controls, was tested in triplicate. The test substance was tested at the following concentrations:

Experiment I & Ia: 3; 10; 33; 100; 333; 1000; 2500; and 5000 µg/plate
Experiment II: 10; 33; 100; 333; 1000; 2500; and 5000 µg/plate

Precipitation of the test substance in the overlay agar in the test tubes as well as on the incubated agar plates was observed from 333 to 5000 µg/plate. The undissolved particles had no influence on the data recording. The plates incubated with the test substance showed normal background growth up to 5000 µg/plate with and without S9 mix in all strains used.

No toxic effects, evident as a reduction in the number of revertants (below the indication factor of 0.5), occurred in the test groups with and without metabolic activation. No substantial increase in revertant colony numbers of any of the five tester strains was observed following treatment with fluopicolide, at any dose level, neither in the presence nor absence of metabolic activation (S9 mix). There was also no tendency of higher mutation rates with increasing concentrations in the range below the generally acknowledged border of biological relevance.

Appropriate reference mutagens were used as positive controls and showed a distinct increase of induced revertant colonies.

In conclusion, it can be stated that during the described mutagenicity test and under the experimental conditions reported, the test substance did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used.

Therefore, fluopicolide is considered to be non-mutagenic in this *Salmonella typhimurium* reverse mutation assay.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 (BCS-AM59797) (fluopicolide)
Purity:	98.2%
Batch no.:	2016-012208

2. Vehicle and/or positive control

Vehicle:	DMSO (purity >99%, concentration not given)
Positive control:	Without S9 mix:
	Na-azide: TA 100, TA 1535
	4-nitro-o-phenylene-diamine (4-NOPD): TA 1537, TA 98
	Methyl methane sulfonate (MMS): TA 102
	With S9 mix:
	2-Aminoanthracene: TA 98, TA 100, TA 1535, TA 1537, TA 102

3. Activation:

Due to the limited capacity for metabolic activation of potential mutagens in *in vitro* methods an exogenous metabolic activation system is necessary.

Phenobarbital/β-naphthoflavone induced rat liver S9 were used as the metabolic activation system. The S9 was prepared and stored according to the currently valid version of the SOP for rat liver S9 preparation. Each batch of S9 was routinely tested for its capability to activate the known mutagens benzo[a]pyrene and 2-aminoanthracene in the Ames test. Furthermore for each S9 batch a sterility test and the determination of the protein concentration were performed.

The protein concentration of the S9 preparation was 32.3 mg/mL (Lot. No.: 090217B) in all experiments.

S9 mix:

An appropriate quantity of S9 supernatant is thawed and mixed with S9 cofactor solution, to result in a final concentration of approx. 10% v/v in the S9 mix. Cofactors are added to the S9 mix to reach the following concentrations in the S9 mix:

8 mM $MgCl_2$
33 mM KCl
5 mM glucose-6-phosphate
4 mM NADP
in 100 mM sodium-ortho-phosphate-buffer, pH 7.4.

The S9 cofactor solution was prepared freshly and sterile-filtrated before the S9 supernatant was added.

During the experiment, the S9 mix is stored in an ice bath. The S9 mix preparation is performed according to Ames *et al* .(1977)[16].

[16] Ames, B.N., J. McCann, and E. Yamasaki (1977) Methods for detecting carcinogens and mutagens with the Salmonella/mammalian microsome mutagenicity test In: B.J. Kilbey et al. (Eds.) Handbook of Mutagenicity Test Procedures Elsevier, Amsterdam, 1-17

4. Test organisms:

The histidine dependent strains are derived from *S. typhimurium* strain LT2 through mutations in the histidine locus. Additionally due to the "deep rough" (rfa-) mutation they possess a faulty lipopolysaccharide envelope, which enables substances to penetrate the cell wall more easily. A further mutation (deletion of the uvrB gene) causes an inactivation of the excision repair system. The latter alteration also includes a deletion in the nitrate reductase and biotin genes. In the strains TA 98, TA 100, and TA 102 the R-factor plasmid pKM 101 carries umu DC analogous genes that are involved in error-prone repair and the ampicillin resistance marker. The strain TA 102 does not contain the uvrB-mutation. Additionally, TA 102 contains the multicopy plasmid pAQ1, which carries the hisG428 mutation and a tetracycline resistance gene. TA 102 contains the ochre mutation in the hisG gene.

The mutations of the bacterial strains used in this study are described in Table 5.4.1- 12.

Table 5.4.1- 12: Salmonella typhimurium strains

	Salmonella typhimurium	
Strains	**Genotype**	**Type of mutations indicated**
TA 1537	his C 3076; rfa⁻; uvrB⁻	frame shift mutations
TA 98	his D 3052; rfa⁻; uvrB⁻; R-factor	"
TA 1535	his G 46; rfa⁻; uvrB⁻	base-pair substitutions
TA 100	his G 46; rfa⁻; uvrB⁻; R-factor	"
TA 102	his G 428; rfa⁻; uvrB⁻; R-factor	"

Regular checking of the properties of the *Salmonella typhimurium* strains regarding the membrane permeability, ampicillin resistance; UV sensitivity and amino acid requirement as well as normal spontaneous mutation rates is performed at Envigo CRS GmbH according to Ames *et al.* (1977)[16] and Maron and Ames (1983)[14]. In this way it is ensured that the experimental conditions set down by Ames are fulfilled.

The bacterial strains TA 1535, TA 1537, TA 98, TA 100, and TA 102 were obtained from Trinova Biochem GmbH (35394 Gießen, Germany).

5. Test substance concentrations used:

In the pre-experiment and the repeated experiment Ia the concentration range of the test item was 3 – 5000 µg/plate. The pre-experiment is reported as experiment I. Since no toxic effects, but precipitation of the test substance was observed in experiment I, seven concentrations were tested in experiment II. 5000 µg/plate were chosen as maximal concentration.

The concentration range included two logarithmic decades. The following concentrations were tested:

Experiment I & Ia: 3; 10; 33; 100; 333; 1000; 2500; and 5000 µg/plate
Experiment II: 10; 33; 100; 333; 1000; 2500; and 5000 µg/plate.

B. Test performance

Experimental phase: April 25 to May 23, 2017

1. Pre-experiment for toxicity

To evaluate the toxicity of the test substance a pre-experiment was performed with all strains used. Eight concentrations were tested for toxicity and mutation induction with each three plates. The experimental conditions in this pre-experiment were the same as described for the experiment I below (plate incorporation test).

The pre-experiment is reported as main experiment I, since the acceptance criteria are met.

Since for the positive control of strain TA 102 with S9 mix the acceptance criteria were not met, this part of experiment I was repeated. The experimental conditions in the repeated experiment Ia were the same as in the pre-experiment.

2. Mutagenicity test

For each strain and dose level, including the controls, three plates were used.

The following materials were mixed in a test tube and poured onto the selective agar plates:

Experiment I (Plate Incorporation):

- 100 µL test solution at each dose level (solvent or reference mutagen solution (positive control)),
- 500 µL S9 mix (for test with metabolic activation) or S9 mix substitution buffer (for test without metabolic activation),
- 100 µL bacteria suspension (cf. test system, pre-culture of the strains),
- 2000 µL overlay agar

Experiment II (pre-incubation):

In the pre-incubation assay 100 µL test solution (solvent or reference mutagen solution (positive control)), 500 µL S9 mix / S9 mix substitution buffer and 100 µL bacterial suspension were mixed in a test tube and incubated at 37 °C for 60 minutes. After pre-incubation 2.0 mL overlay agar (45 °C) was added to each tube. The mixture was poured on minimal agar plates.

After solidification the plates were incubated upside down for at least 48 hours at 37 °C in the dark (De Serres and Shelby[15]).

In parallel to each test a sterile control of the test substance was performed and documented in the raw data. Therefore, 100 µL of the stock solution, 500 µl S9 mix / S9 mix substitution buffer were mixed with 2.0 mL overlay agar and poured on minimal agar plates.

Data recording:

The colonies were counted using a validated computer system (cf. 3.8, major computerized systems), which was connected to a PC with printer to print out the individual values, the means from the plates for each concentration together with standard deviations and enhancement factors as compared to the spontaneous reversion rates (see tables of results).

Due to precipitation of the test substance the colonies were partly counted manually.

3. Statistics

According to the OECD guideline 471, a statistical analysis of the data is not mandatory.

4. Acceptance / assessment criteria:

The *Salmonella typhimurium* reverse mutation assay is considered acceptable if it meets the following criteria:

- Regular background growth in the negative and solvent control.
- The spontaneous reversion rates in the negative and solvent control are in the range of our historical data.
- The positive control substances should produce an increase above the threshold of twice (strains TA 98, TA 100, and TA 102) or thrice (strains TA 1535 and TA 1537) the colony count of the corresponding solvent control.
- A minimum of five analysable dose levels should be present with at least three dose levels showing no signs of toxic effects, evident as a reduction in the number of revertants below the indication factor of 0.5.

5. Evaluation of results

A test substance is considered as a mutagen if a biologically relevant increase in the number of revertants exceeding the threshold of twice (strains TA 98, TA 100, and TA 102) or thrice (strains TA 1535 and TA 1537) the colony count of the corresponding solvent control is observed.

A dose dependent increase is considered biologically relevant if the threshold is exceeded at more than one concentration.

An increase exceeding the threshold at only one concentration is judged as biologically relevant if reproduced in an independent second experiment.

A dose dependent increase in the number of revertant colonies below the threshold is regarded as an indication of a mutagenic potential if reproduced in an independent second experiment. However, whenever the colony counts remain within the historical range of negative and solvent controls such an increase is not considered biologically relevant.

II. Results and Discussion

A. Mutation assays

The test substance fluopicolide was assessed for its potential to induce gene mutations according to the plate incorporation test (experiment I and Ia) and the pre-incubation test (experiment II) using *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, TA 100, and TA 102.

The assay was performed in three independent experiments both with and without liver microsomal activation. Each concentration, including the controls, was tested in triplicate.

The test substance was tested at the following concentrations:

Pre-Experiment/Experiment I: 3; 10; 33; 100; 333; 1000; 2500; and 5000 µg/plate
Experiment II: 10; 33; 100; 333; 1000; 2500; and 5000 µg/plate

Precipitation of the test substance in the overlay agar in the test tubes as well as on the incubated agar plates was observed from 333 to 5000 µg/plate. The undissolved particles had no influence on the data recording. The plates incubated with the test substance showed normal background growth up to 5000 µg/plate with and without S9 mix in all strains used.

No toxic effects, evident as a reduction in the number of revertants (below the indication factor of 0.5), occurred in the test groups with and without metabolic activation. No substantial increase in revertant colony numbers of any of the five tester strains was observed following treatment with fluopicolide at any dose level, neither in the presence nor absence of metabolic activation (S9 mix). There was also no tendency of higher mutation rates with increasing concentrations in the range below the generally acknowledged border of biological relevance.

Appropriate reference mutagens were used as positive controls and showed a distinct increase of induced revertant colonies.

An overview of the results is given in Table 5.4.1- 13 to Table 5.4.1- 16.

Table 5.4.1- 13: Summary of experiment I

Test Group	Dose Level (µg/plate)	Revertant Colony Counts (Mean±SD)				
		TA 1535	TA 1537	TA 98	TA 100	TA 102
Without metabolic activation (-S9)						
Fluopicolide	3	14±3	6±3	23±4	168±10	526±20
	10	12±5	9±5	24±6	163±12	553±20
	33	13±3	10±3	26±9	166±16	496±6
	100	10±1	8±1	24±3	164±12	499±10
	333	12±2 P	9±3 P	30±4 P	163±10 P	503±11 P
	1000	12±2 P	9±2 P	28±3 P	177±9 P	516±14 P
	2500	9±1 PM	5±2 PM	21±2 PM	162±8 PM	421±29 PM
	5000	8±3 PM	6±1 PM	18±4 PM	163±18 PM	471±48 PM
DMSO	-	11±5	8±2	24±10	166±11	525±10
Untreated	-	11±3	6±1	24±4	198±14	536±9
Positive control						
NaN_3	10	1290±93	NA	NA	2240±115	NA
4-NOPD	10	NA	NA	248±7	NA	NA
	50	NA	76±9	NA	NA	NA
MMS	2.0 µL	NA	NA	NA	NA	4330±251
Historical solvent control mean	-	12±2.3	10±2.3	25±4.4	156±26.0	463±51.0
Historical control range	-	6-25	6-19	13-43	78-209	320-534

Test Group	Dose Level (µg/plate)	Revertant Colony Counts (Mean±SD)				
		TA 1535	TA 1537	TA 98	TA 100	TA 102
With metabolic activation (+S9)						
Fluopicolide	3	15±6	9±3	33±8	128±1	691±6
	10	9±1	9±7	29±5	146±24	693±25
	33	14±5	11±4	31±4	158±25	682±63
	100	12±4	12±6	30±8	152±4	630±40
	333	12±6 P	15±3 P	34±4	141±10 P	679±27 P
	1000	12±5 P	12±3 P	31±6 P	151±1 P	670±18 P
	2500	10±3 P	12±3 P	40±2 P	139±9 P	613±11 P
	5000	13±3 P	8±4 PM	31±5 PM	144±9 PM	581±45 PM
DMSO	-	11±6	10±3	33±4	136±5	632±3
Untreated	-	12±3	13±6	36±4	158±19	667±7
Positive control						
2-AA	2.5	443±23	138±29	4507±509	4072±348	NA
	10.0	NA	NA	NA	NA	1135±38
Historical solvent control mean	-	12±2.5	13±3.5	34±6.2	148±32.3	571±71.1
Historical control range	-	7-26	7-30	16-58	73-208	325-652

NaN_3 = sodium azide; 2-AA = 2-aminoanthracene; MMS = methyl methane sulfonate; 4-NOPD = 4-nitro-o-phenylene-diamine
NA: not applicable
P: precipitate
M: manual count
Historical control data based on approx. 600 experiments (in case of TA 102 the historical data are based on approx. 150 experiments) from November 2014 until November 2016.

Table 5.4.1- 14: Summary of experiment Ia (with metabolic activation; +S9)

Test Group	Dose Level (µg/plate)	Revertant Colony Counts (Mean±SD) TA 102
Fluopicolide	3	591±42
	10	536±30
	33	548±31
	100	578±37
	333	570±24 P
	1000	581±5 P
	2500	546±4 P
	5000	576±35 PM
DMSO	-	603±12
Untreated	-	627±23
Positive control 2-AA	10.0 µg	1409±92
Historical solvent control mean	-	571±71.1
Historical control range		325-652

2-AA = 2-aminoanthracene
P: precipitate
M: manual count
Historical control data based on approx. 150 experiments from November 2014 until November 2016

Table 5.4.1- 15: Summary of experiment II (pre-incubation test)

Test Group	Dose Level (µg/plate)	Revertant Colony Counts (Mean±SD) TA 1535	TA 1537	TA 98	TA 100	TA 102
Without metabolic activation (-S9)						
Fluopicolide	10	10±1	11±3	25±6	135±8	470±43
	33	11±2	10±4	27±3	135±11	422±21
	100	13±3	10±1	21±1	133±6	476±29
	333	11±2 P	10±1 P	26±6 P	138±20 P	448±3 P
	1000	11±3 PM	11±4 P	22±2 PM	147±10 P	445±17 P
	2500	13±3 PM	8±2 PM	29±3 PM	139±10 PM	435±6 PM
	5000	14±3 PM	9±1 PM	22±5 PM	148±24 PM	410±17 PM
DMSO	-	13±1	11±4	25±4	151±18	458±47
Untreated	-	9±4	11±1	24±5	209±5	458±23
Positive control						
NaN3	10	1261±35	NA	NA	2031±132	NA
4-NOPD	10	NA	NA	328±15	NA	NA
	50	NA	107±11	NA	NA	NA
MMS	2.0 µl	NA	NA	NA	NA	2887±129

Test Group	Dose Level (µg/plate)	Revertant Colony Counts (Mean±SD) TA 1535	TA 1537	TA 98	TA 100	TA 102
With metabolic activation (+S9)						
Fluopicolide	10	14±2	15±1	43±10	112±10	690±64
	33	12±4	14±1	39±9	102±3	628±27
	100	14±3	12±4	40±3	122±27	620±85
	333	15±1 P	15±3 P	44±7 P	115±18 P	680±8 P
	1000	13±2 PM	12±2 PM	46±4 P	122±8 P	691±15 P
	2500	12±2 PM	14±4 PM	29±5 PM	111±14 PM	703±14 PM
	5000	12±4 PM	8±2 PM	25±4 PM	118±11 PM	706±9 PM
DMSO	-	13±3	14±4	43±2	112±3	646±7
Untreated	-	17±3	23±5	40±6	110±10	659±30
Positive control 2-AA	2.5	348±33	176±18	4733±364	3089±322	NA
	10.0	NA	NA	NA	NA	1467±30

NaN3 = sodium azide; 2-AA = 2-aminoanthracene; MMS = methyl methane sulfonate; 4-NOPD = 4-nitro-o-phenylene diamine
NA: not applicable
P: precipitate
M: manual count

III. Conclusion

In conclusion, it can be stated that during the described mutagenicity test and under the experimental conditions reported, the test substance did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used. Therefore, fluopicolide is considered to be non-mutagenic in this *Salmonella typhimurium* reverse mutation assay.

Assessment and conclusion by applicant:

The study was conducted according to OECD TG 471 and is valid and acceptable to assess the mutagenic potential of fluopicolide in *S-typhimurium* and *E. Coli*. Fluopicolide is not mutagenic in bacterial cells under the conditions of this study when tested up to precipitating concentrations (+/- S9), either by base pair changes or frameshift mutations.

Data Point:	KCA 5.4.1/06
Report Author:	[redacted]
Report Year:	2004
Report Title:	In vitro Chinese hamster lung V79 cells chromosome aberration assay Code: AE C638206 00 1C99 0005
Report No:	C008174
Document No:	M-197260-02-1
Guideline(s) followed in study:	Directive 92.69/EEC, Annex V, B10; OECD 473 (1997); US-EPA OPPTS 870.5375 (1998);
Deviations from current test guideline:	Deviations from the current OECD guideline (473, 2016): - Cytotoxicity was not measured using the parameters of relative population doubling (RPD) or relative increase in cell count (RICC) - Only 25-100 metaphases, instead of the currently required 300 metaphases, were analysed The deviation(s) are considered not to compromise the results and outcome of the study.
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

This study assessed the potential of technical fluopicolide (mixture of Batch No. PP/241024/2 & PP241067/1) to induce chromosome aberrations in Chinese hamster lung V79 cells *in vitro*. For each experiment two cell cultures were used.

Following preliminary toxicity testing, two independent chromosome aberration tests were conducted in the absence and one in the presence of an exogenous metabolic activation system (S9-mix) using a range of concentrations of the test compound. Positive and vehicle (DMSO) cultures were included in each assay. Duplicate cell cultures per dose level were used. The cells sampled 20 hours after the start of treatment and examined for chromosome aberrations.

The test compound was dissolved in DMSO and tested at the following concentrations:

First experiment with 3 h treatment time:

without S9-mix: 3.2, 6.3, 12.5, 25.0*, 50.0*, 75.0* and 100.0* µg/mL
with S9-mix: 3.2, 6.3, 12.5, 25.0*, 50.0*, 75.0* and 100.0* µg/mL

Second experiment with 20 h treatment time:

without S9-mix: 0.1, 0.2, 0.4, 0.8, 1.6*, 3.2* and 6.3* µg/mL

* = slides evaluated

The concentration ranges were based on the results of preliminary testing for solubility and toxicity. The test substance produced a distinct lowering of the mitotic index in the first main experiment at a concentration of 100 µg/mL in the absence of metabolic activation and at the dose levels of 75 and 100 µg/mL in the presence of metabolic activation. In the second main experiment cytotoxicity as a reduction of the mitotic index was observed at dose levels of 3.2 µg/mL and above.

In the first main experiment the test substance induced a statistically significant increase in the number of chromosome aberrations at a concentration of 100 µg/mL in the absence and in the presence of metabolic activation (mitotic indices 38.5% and 48.7% respectively). In the second main experiment without S9-mix a statistically significant increase of the aberration rate was observed at a dose level of 3.2 µg/mL (corresponding to a mitotic index of 63.8%) and at a concentration of 6.3 µg/mL (mitotic index of 22.4%).

Because of the clear positive results at one concentration with a mitotic index higher than 50% of the solvent control value and considering the low cytotoxicity in the microwell plates, fluopicolide was judged to be clastogenic. Appropriate reference mutagens used as positive controls showed a significant increase in chromosome aberrations, thus indicating the sensitivity of the assay, and the efficacy of the S9-mix.

Fluopicolide was clastogenic in this *in vitro* chromosome aberration assay with V79 Chinese hamster lung cells in both the presence and absence of metabolic activation.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 (fluopicolide), technical
Purity:	97.8% (w/w)
Batch no.:	mixture of PP/241024/2 & PP241067/1

2. Vehicle and/or positive control

Vehicle:	DMSO
Positive control:	Non-activation:
	Ethyl methane sulfonate, dissolved in cell culture medium on the day of treatment:
	final concentration: 1.5 mg/mL (3 h treatment)
	final concentration: 0.4 mg/mL (20 h treatment)
	Activation:
	Cyclophosphamide, dissolved in cell culture medium on the day of treatment, final concentration in cell culture medium: 2.5 µg/mL

3. Activation:

The S9 fraction was prepared by the testing facility according to Ames *et al.* (1975)[13]. Male Sprague Dawley rats (200-300 g), supplied by Harlan Winkelmann, Gartenstrasse 27, 33178 Borchen, Germany, received a single intraperitoneal injection of Aroclor 1254 (500 mg/kg bw) 5 days before killing. The livers were removed from at least 5-6 animals and, using cold sterile solutions at approx. 0 to 4 °C and glassware, were then pooled and washed in approx. 150 mM KCl (approx. 1 mL/g wet liver). The washed livers were cut into small pieces and homogenized in three volumes of KCl. The homogenate was centrifuged at approx. 9000 g for 10 minutes. The supernatant, the S9 fraction, was divided into small portions, rapidly frozen and stored at approx. –80 °C for not longer than six months. The protein content was determined for every batch. Also for every batch of S9 an independent validation was performed with a minimum of two different mutagens, e.g. 2-aminoanthracene and dimethylbenzanthracene, to confirm metabolic activation by microsomal enzymes.

Preparation of S9-mix:

Sufficient S9 fraction was thawed to room temperature immediately before each test. An appropriate quantity of S9 fraction (batch no. 99/6, protein concentration 27.5 g/L) was mixed with S9 cofactor solution to yield a final protein concentration of 0.3 mg/mL in the cultures which was kept on ice until used. This preparation is termed S9-mix. The concentrations of the different cofactors of the S9-mix were:

8 mM $MgCl_2$
33 mM KCl
5 mM glucose-6-phosphate
5 mM NADP
100 mM phosphate buffer pH 7.4

4. Cell cultures:

Large stocks of the mycoplasma-free V79 cell line are stored in liquid nitrogen in the cell bank of "Genetic Toxicology", thus permitting repeated use of the same cell culture batch for numerous experiments. The identical characteristics of the cells ensure comparability of the experimental parameters.

5. Culture Medium:

Thawed stock cultures were kept at approx. 37 °C and approx. 4% CO_2 in 175 cm^2 plastic flasks. About 5 x 10^5 to 1 x 10^6 cells were seeded into each flask in 30 mL of MEM-medium supplement with approx. 10% (v/v) FCS (foetal calf serum) containing approx. 2 mM L-glutamine and approx. 0.1% (w/v) neomycinsulfate. The cells were subcultured twice a week.

6. Test substance concentrations used:

Table 5.4.1- 16 gives an overview of the test substance concentrations.

Table 5.4.1- 16: Test substance concentrations

Group	1st experiment			2nd experiment		
	S9 mix	Concentration (µg/mL)	Treatment time (h)	S9 mix	Concentration (µg/mL)	Treatment time (h)
Solvent control	- / +	0	3	-	0	20
Fluopicolide	- / +	3.2	3	-	0.1	20
	- / +	6.3	3	-	0.2	20
	- / +	12.5	3	-	0.4	20
	- / +	25.0*	3	-	0.8	20
	- / +	50.0*	3	-	1.6*	20
	- / +	75.0*	3	-	3.2*	20
	- / +	100	3	-	6.3*	20
Ethyl methane		1500	3	-	400	20
Cyclophosphamide	+	2.5	3	/	/	/

* = 1 slide evaluated

B. Test performance

Experimental phase: April 17 to May 19, 2000

1. Preliminary assay

The concentrations for the mutagenicity assay are based on the results of the toxicity experiment.

For non-toxic, freely soluble test substances, the top dose is either 10 mM or 5000 µg/mL according to international testing guidelines. For relatively insoluble test substances that are not toxic at concentrations lower than the insoluble concentration, the highest dose used is a concentration above the limit of solubility in the final culture medium after the end of the treatment period. For toxic compounds, the highest dose level is selected to reduce survival to approx. 20-50%, and/or the mitotic index to approx. 50% of the corresponding solvent control.

A preliminary toxicity test was undertaken in order to select appropriate dose levels for the cytogenetic assay. Cell cultures were subjected to the same treatment conditions as in the main experiment. Cytotoxic effects were determined by photometric measurement of V79 cell cultures grown in microwell plates (approx. 3125 cells/cm^3) and stained with crystal violet. The relative cell density in the microwell plates was similar to that in the Quadriperm® dishes.

The test included the following treatments:

Solvent control: the maximum final concentration of organic solvents was approx. 1% (v/v).

Test substance: the highest dose level for the preliminary toxicity test was determined by the solubility of the test compound and equated to the international maximum of 10 mM.

Treatments were performed both in the presence and absence of the S9-mix metabolic activation system using a single cell culture at each test point.

2. Cytogenetic Assay

Two independent experiments were conducted in the presence of S9-mix and one test in its absence using duplicate cultures of cells seeded onto slides (i.e. 2 per dose level) and at least three dose levels.

In the first experiment cells were treated for 3 hours in both the presence and absence of S9-mix whereas in the second assay they were treated for 20 hours in the absence of S9-mix alone. In both experiments the cells were sampled 20 hours after the start of treatment as were the concurrent solvent and positive control cultures. Colcemide was added to each culture 2 hours before sampling in order to arrest cell division.

Chromosome preparations were made, fixed, stained and examined. However, where clearly positive results were obtained in the first experiment, those from the second assay were not examined. Where equivocal or negative results were obtained in the first experiment, modifications to the testing procedure were included in order to clarify the result.

Before treatment, the pH values and osmolality of the treatment medium were determined. If necessary the pH was adjusted to pH 7.3 with NaOH or HCl. Any effects on the osmolality during the study were described in the study report.

Exponentially growing cultures which were more than 50% confluent were trypsinated by an approx. 0.25% (v/v) trypsin solution ready for use (supplied by Gibco) and a single cell suspension (culture) was prepared. The trypsin concentration was approx. 0.25% (v/v) in Ca-Mg-free salt solution. Two slides were placed in Quadriperm® dishes which were then seeded with cells to yield 3-4 x 10^4 cells/slide. Thus for each dose level and treatment time, duplicate cultures slides are used. The Quadriperm® dishes contain 6 mL MEM with approx. 10% (v/v) FCS.

After 48 h, the medium was replaced with one containing approx. 10% (v/v) FCS and the test substance, or positive control, or solvent and in the presence of metabolic activation additionally 2% (v/v) S9-mix. For the 3 hour treatment time, the medium was replaced by normal medium following two rinses. In the second experiment the cells were exposed to the treatment medium without S9-mix for 20 h.

18 h after the start of the treatment, Colcemide was added (approx. 0.05 µg/mL culture medium) to the cultures to arrest mitosis and 2 h later (20 h after the start of treatment) metaphase spreads were prepared as follows:

The cultures were made hypotonic by adding about 5 mL of approx. 0.075 M potassium chloride solution at around 37 °C. The cells were then incubated for 20 minutes at approx. 37 °C. The next step was the addition of 2 mL fixative.

Then the liquid was replaced by 6 mL fixative (methanol: glacial acetic acid, 3:1). After 10 minutes the procedure was repeated. After at least another 10 minutes, the slides were taken out and air-dried for 24 hours. The chromosomes were stained as follows:

- staining for 10 minutes in approx. 2% (w/v) orcein solution
- rinsing 3 times in distilled water
- rinsing twice in acetone
- brief rinsing in acetone/xylene
- 2 minutes in acetone/xylene
- 5 minutes in xylene
- 10 minutes in xylene
- embedding in Entellan® or Corbit®

3. Evaluation of data:

Analysis of metaphases:

The slides were coded and 25-100 metaphases per experimental group and cell culture were examined. The set of chromosomes was examined for completeness and the various chromosomal aberrations were assessed and classified. Only metaphases with 22 +/- 2 chromosomes are included in the analysis. The metaphases were examined for the following aberrations: chromatid gap, chromosome gap, chromatid break, chromosome break, minute, double minute, chromatid deletion, chromosome deletion, chromatid exchanges including intra-changes, chromosome exchanges including intra-changes, dicentrics, pulverization and ring formation. Furthermore the incidence of polyploid metaphases was determined in 1000 cells of each cell culture. Additionally the mitotic index was determined by counting the number of cells undergoing mitosis in a total of 1000 cells. The mitotic index is expressed as a percentage.

After the metaphases had been evaluated, the code was broken. For each experiment the results from the dose groups were compared with those of the control group and the positive control at each sampling time.

4. Criteria for a valid assay:

The assay was considered valid if the following criteria are met:

- The solvent control data were within the laboratory's normal control range for the spontaneous mutant frequency.
- The positive controls induced increases in the mutation frequency which were both statistically significant and within the laboratory's normal range.

5. Criteria for a positive response:

The evaluation of the results was performed as follows:

- The test substance is classified as clastogenic if it induces a statistically significant increase in the aberration rate (without gaps) with one or more of the concentrations tested as compared with the solvent controls.
- The test substance is classified as clastogenic if there is a reproducible concentration-related increase in the aberration rate (without gaps).
- The test substance is classified as not clastogenic if the tests are negative both with and without metabolic activation.

6. Statistics

The Biometry of the results was performed with a one-sided Fisher-Exact test.

II. Results and Discussion

A. Preliminary cytotoxicity assay

Fluopicolide was suspended in DMSO at a stock concentration of 382 mg/mL. Evaluation of the solubility of that suspension in cell culture medium showed that 3820 µg/mL was the highest practicable concentration and produced precipitate. This concentration corresponds to 10 mM, which is the international top dose level for these studies.

Accordingly, the preliminary toxicity study was carried out using a maximum concentration of 3820 µg/mL and a range of lower dose levels down to 10 µg/mL.

Following treatment in the absence of S9 metabolic activation survival declined in a dose-dependent manner reaching 44.5% of the solvent control value (after a treatment time of 3 h) and 20.4% of the solvent control value (after a treatment time of 20 h) at the highest dose level, 3820 µg/mL.

In the presence of S9 metabolic activation survival was dose-dependently reduced after a treatment time of 3 h to 55.4% of the solvent control value at the highest dose level, 3820 µg/mL.

In a dose range of 120 to 3820 µg/mL at a treatment time of 3 hours and in a dose range of 12.1 to 3820 µg/mL at the treatment time of 20 hours the cells survived, but so extremely damaged, that no scorable metaphases were found.

Based in these results a concentration of 100 µg/mL (stock solution in DMSO of 10 mg/mL) were chosen as maximum dose level for a treatment time of 3 hours in the absence and in the presence of S9-mix in the first main experiment. For the fixation interval of 20 hours 6.3 µg/mL were determined as the top dose level in the second main experiment.

Before treatment, the pH values and osmolality of the treatment media were determined. The addition of test substance solutions did not have any effect on these parameters.

B. Cytogenetic assay

Mitotic index:

In the main experiments cytotoxicity was also evaluated by treatment of cells seeded in microwell plates. At the 3 hours treatment time survival was reduced in a dose-dependent manner reaching 68.4% of the solvent control value without S9-mix and 73.1% of the solvent control with S9-mix at a dose level of 100 µg/mL. At 20 hours treatment time in the absence of S9-mix 58.9% of the solvent control survived after treatment with at the highest concentration tested, 6.3 µg/mL.

The test substance produced a distinct lowering of the mitotic index in the first main experiment at a concentration of 100 µg/mL in the absence of metabolic activation and at the dose levels of 75 and 100 µg/mL in the presence of metabolic activation. In the second main experiment cytotoxicity as a reduction of the mitotic index was observed at dose levels of 3.2 µg/ml and above.

Treatment with the test substance did not cause any relevant increase in the number of polyploid cells as compared with the solvent controls.

Chromosome aberrations:

The test substance fluopicolide was assessed for its potential to induce chromosome aberrations (clastogenicity) in two independent *in vitro* experiments without metabolic activation and one experiment with metabolic activation. There was a statistically significant increase in the absolute aberration numbers 3 h after the start of the treatment with 100 µg/mL in the absence and in the presence of S9-mix (data not shown). Because of a distinct lowering of the mitotic index at these dose levels (38.3% without S9-mix and 48.7% with S9-mix), the slides of the second main experiment were also evaluated. At the sampling time of 20 h without S9-mix at the concentrations 3.2 µg/mL (corresponding to a mitotic index of 63.8%) and at a concentration of 6.3 µg/mL (mitotic index of 22.4%) the absolute aberration numbers were statistically significantly increased.

Because of the clear positive results at one concentration (3.2 µg/mL) with a mitotic index higher than 50% of the solvent control value and considering the low cytotoxicity in the microwell plates, fluopicolide was judged to be clastogenic.

The sensitivity of the test system was also demonstrated by the enhanced mutation frequency in the cell cultures treated with the positive control compounds.

Table 5.4.1- 17 and Table 5.4.1- 18 give an overview of the study results.

Table 5.4.1- 17: Relative mitotic indices and mean percentage of aberrant V79 cells, including and excluding gaps – Experiment I

Treatment	Concentration (µg/ml)	Treatment time (h)	Relative mitotic index percent (1000 cells)	Percent aberrant cells Including gaps	Percent aberrant cells Excluding gaps	Percent aberrant cells exchanges
Without metabolic activation (-S9)						
Fluopicolide	100	3	38.3	10.5	8.0	0.5
	75	3	76.6	2.5	0.5	0.0
	50	3	81.3	1.5	0.0	0.0
	25	3	110.3	1.0	0.5	0.0
Solvent control	-	3	100.0	1.5	1.0	0.0
Positive control Ethyl methane sulfonate	1500	3	92.5	22.0	20.0	17.0

Treatment	Concentration (µg/ml)	Treatment time (h)	Relative mitotic index percent (1000 cells)	Percent aberrant cells Including gaps	Percent aberrant cells Excluding gaps	Percent aberrant cells exchanges
With metabolic activation (+S9)						
Fluopicolide	100	3	48.7	10.5	7.0	1.0
	75	3	43.6	6.5	3.0	0.5
	50	3	114.1	2.0	0.0	0.0
	25	3	106.4	2.5	2.5	0.5
Solvent control	-	3	100.0	2.5	1.5	0.0
Positive control Cyclophosphamide	2.5	3	123.1	26.0	19.0	8.0

No statistical analyses were performed for the relative / percentage values

Table 5.4.1- 18: Relative mitotic indices and mean percentage of aberrant V79 cells, including and excluding gaps – Experiment II, without metabolic activation (-S9)

Treatment	Concentration (µg/ml)	Treatment time (h)	Relative mitotic index percent (1000 cells)	Percent aberrant cells Including gaps	Percent aberrant cells Excluding gaps	Percent aberrant cells exchanges
Fluopicolide	6.3	20	22.4	17.0	12.5	0.0
	3.2	20	63.8	13.5	6.5	2.0
	1.6	20	105.2	0.5	0.0	0.0
Solvent control	-	20	100.0	1.0	0.0	0.0
Positive control Ethyl methane sulfonate	400	20	72.4	14.0	10.0	7.5

III. Conclusion

Fluopicolide was clastogenic in both the presence and absence of metabolic activation in this *in vitro* chromosome aberration assay with V79 Chinese hamster lung cells.

Assessment and conclusion by applicant:

The study was conducted according to OECD TG 473 and is valid and acceptable to assess the potential of fluopicolide to induce chromosome aberrations in Chinese Hamster Lung V79 cells *in vitro*. Fluopicolide is not clastogenic under the conditions of this assay (+/- S9).

Data Point:	KCA 5.4.1/07
Report Author:	[redacted]
Report Year:	2001
Report Title:	AE C638206: In vitro mammalian chromosome aberration test in human lymphocytes
Report No:	C011815
Document No:	M-201582-01-1
Guideline(s) followed in study:	OECD 473 (1997); US-EPA 712-C-98-223.; OPPTS 870.5375 (1998)
Deviations from current test guideline:	Deviations from the current OECD guideline (473, 2016): - Cytotoxicity was not measured using the parameters of relative population doubling (RPD) or relative increase in cell count (RICC) - Only 25-100 metaphases, instead of the currently required 300 metaphases, were analysed The deviation(s) are considered not to compromise the results and outcome of the study.
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

This study was performed to assess the ability of fluopicolide to induce chromosomal aberrations in human lymphocytes cultured *in vitro*.

Human lymphocytes in whole blood culture, were stimulated to divide by addition of phytohaemagglutinin, and exposed to the test substance both in the presence and absence of S9 mix derived from rat livers. Solvent and positive control cultures were also prepared. Two hours before the end of the incubation period, cell division was arrested using Colcemid®, the cells harvested and slides prepared, so that metaphase cells could be examined for chromosomal damage.

In order to assess the toxicity of fluopicolide to cultured human lymphocytes, the mitotic index was calculated for all cultures treated with the test substance and the solvent control. On the basis of these data, the following concentrations were selected for metaphase analysis:

First test:

- Without S9 mix - 3 hours treatment, 18 hours recovery: 19.53, 78.13 and 156.25 µg/mL.
- With S9 mix - 3 hours treatment, 18 hours recovery: 78.13, 312.5 and 625 µg/mL.

Second test:

- Without S9 mix - 21 hours continuous treatment: 1.22, 9.77 and 19.53 µg/mL.
- With S9 mix - 3 hours treatment, 18 hours recovery: 39.06, 156.25 and 312.5 µg/mL.

In both the absence and presence of S9 mix, fluopicolide caused no statistically significant increase in the proportion of metaphase figures containing chromosomal aberrations, at any dose level, when compared with the solvent control, in either test.

A quantitative analysis for polyploidy was made in cultures treated with the negative control and highest dose level. No increases in the proportion of polyploid cells were seen in either test.

All positive control compounds caused large, statistically significant increases in the proportion of aberrant cells, demonstrating the sensitivity of the test system and the efficacy of the S9 mix.

It is concluded that fluopicolide has shown no evidence of clastogenic activity in this *in vitro* cytogenetic test system, under the experimental conditions described.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 (fluopicolide)
Purity:	95.9%
Batch no.:	OP2050046

2. Vehicle and/or positive control

Vehicle:	DMSO (highest final concentration used for subsequent testing was 625 mg/mL)
Positive control:	Non-activation: Mitomycin (0.1 µg/mL) Activation: Cyclophosphamide (6 µg/mL)

3. Activation:

S9 fraction was prepared from a group of ca. 10 animals (male rat, Sprague-Dawley derived, Charles River UK, 7-8 weeks old, <300 g). Mixed function oxidase systems in the rat livers were stimulated by Aroclor 1254, administered as a single intraperitoneal injection in corn oil at a dosage of 500 mg/kg bw. On the fifth day after injection, following an overnight fasting, the rats were killed and their livers aseptically removed.

The following steps were carried out at 0-4 °C under aseptic conditions. The livers were placed in 0.15 M KCl (3 mL KCl: 1 g liver) before being transferred to a homogeniser. Following preparation, the homogenates were centrifuged at 9000 x *g* for 10 minutes. The supernatant fraction (S9 fraction) was dispensed into aliquots and stored at -80 °C or below until required.

Preparation of S9-mix:

S9 mix contained: S9 fraction (10 % v/v), $MgCl_2$ (8 mM), KCl (33 mM), sodium orthophosphate buffer pH 7.4 (100 mM), glucose-6-phosphate (5 mM), NADP (4 mM). All the cofactors were filter-sterilised before use.

Human blood was collected aseptically from healthy, non-smoking male donors, pooled and diluted with RPMI 1640 tissue culture medium (Sigma) supplemented with 10% foetal calf serum (Globepharm), 1 unit/mL Heparin (CP Pharmaceuticals), 20 I.U./ml penicillin/20 µg/mL streptomycin (Imperial) and 2.0 mM glutamine (Imperial). Aliquots (0.4 mL blood: 4.5 mL medium: 0.1 mL phytohaemagglutinin (Gibco)) of the cell suspension were placed in sterile universal containers and incubated at 37 °C for approx. 48 hours. The cultures were gently shaken daily to re-suspend the cells.

5. Test substance concentrations used:

Test concentrations for the first experiment were 0 (solvent control), 4.88, 9.77, 19.53, 39.06, 78.13, 156.25, 312.5 and 625 µg/mL in tests with and without metabolic activation.

In the second experiment a continuous treatment was used in the absence of S9 mix at test concentrations of 1.22, 2.44, 4.88, 9.77, 19.53, 39.06, 78.13, 156.25, 312.5 and 625 µg/mL and in the presence of S9 mix, a three hour treatment was used, as in the first test at concentrations of 4.88, 9.77, 19.53, 39.06, 78.13, 156.25, 312.5 and 625 µg/mL. The harvest time was at 21 hours for both parts of the test.

On the basis of the toxicity data, the following concentrations were selected for metaphase analysis:

Experiment I without S9 mix - 3 hours treatment, 18 hours recovery: 19.53, 78.13 and 156.25 µg/mL.
Experiment I with S9 mix - 3 hours treatment, 18 hours recovery: 78.13, 312.5 and 625 µg/mL.

Experiment II without S9 mix - 21 hours continuous treatment: 1.22, 9.77 and 19.53 µg/mL.
Experiment II with S9 mix - 3 hours treatment, 18 hours recovery: 39.06, 156.25 and 312.5 µg/mL.

B. Test performance

Experimental phase: September 19 to November 3, 2000

1. Preliminary Assay

After approx. 48 hours, 50 µL aliquots of fluopicolide were added to one set of duplicate cultures to give final concentrations of 4.88, 9.77, 19.53, 39.06, 78.13, 156.25, 312.5 and 625 µg/mL. DMSO, the solvent control, in 50 µL aliquots, was added to two cultures. Mitomycin C at a final concentration of 0.1 mg/mL, was added to duplicate cultures.

Immediately before treatment of the second set of cultures, 1 mL of medium was removed from each culture and discarded. This was replaced with 1 ml of S9 mix, followed by 50 µL aliquots of the various dilutions of fluopicolide, giving the same series of final concentrations as above. DMSO (50 µL) was added to two cultures. Cyclophosphamide was added to duplicate cultures at a final concentration of 6 µg/mL.

Three hours after dosing, the cultures were centrifuged at 500 x *g* for 5 minutes. The cell pellets were rinsed and re-suspended in fresh medium. They were then incubated for a further 18 hours.

Harvesting and fixation:

Two hours before the cells were harvested, mitotic activity was arrested by addition of Colcemid (Sigma) to each culture at a final concentration of 0.1 ng/ml. After 2 hours incubation, each cell suspension was transferred to a conical centrifuge tube and centrifuged for 5 minutes at 500 *g*. The cell pellets were treated with a hypotonic solution (0.075 M KCl pre-warmed at 37 °C). After a 10 minute period of hypotonic incubation at 37 °C the suspensions were centrifuged at 500 *g* for 5 minutes and the cell pellets fixed by addition of freshly prepared cold fixative (3 parts methanol : 1 part glacial acetic acid). The fixative was replaced twice.

Slide preparation:

The pellets were resuspended, then centrifuged at 500 *g* for 5 minutes and finally re-suspended in a small volume of fresh fixative. A few drops of the cell suspensions were dropped onto pre-cleaned microscope slides which were then allowed to air-dry. The slides were then stained in 10 % Giemsa, prepared in buffered water (pH 6.8). After rinsing in buffered water the slides were left to air-dry and then mounted in DPX.

Microscopic examination:

The prepared slides were examined by light microscopy using a low power objective. The proportion of mitotic cells per 1000 cells in each culture was recorded except for positive control treated cultures.

From these results the dose level causing a decrease in mitotic index of approx. 50% of the solvent control value or, if there was no decrease, the maximum achievable concentration was used as the highest dose level for the metaphase analysis. The intermediate and low dose levels were also selected.

The concentration of each positive control compound selected for analysis was the lowest concentration dosed unless a preliminary scan of metaphase figures indicated an insufficient level of aberrant cells. The selected slides were then coded. Metaphase cells were identified using a low power objective and examined at a magnification of x1000 using an oil immersion objective. One hundred metaphase figures were examined, where possible, from each culture. Chromosome aberrations were scored according to the classification of the ISCN (1985). Only cells with 44-48 chromosomes were analysed.

Polyploid and endoreduplicated cells were noted when seen. The vernier readings of all aberrant metaphase figures were recorded. The incidence of polyploid metaphase cells, out of 500 metaphase cells, was determined quantitatively for negative control cultures and cultures treated with the highest dose level of the test substance used in the analysis for chromosomal aberrations.

The number of aberrant metaphase cells in each treatment group was compared with the solvent control value using Fisher's test.

2. Second test

Cultures were initiated and maintained as previously described. In this second test a continuous treatment was used in the absence of S9 mix. In the presence of S9 mix, a three hour treatment was used, as in the first test. The harvest time was at 21 hours for both parts of the test. Concentrations of fluopicolide were as follows:

Without S9 mix: 1.22, 2.44, 4.88, 9.77, 19.53, 39.06, 78.13, 156.25, 312.5 and 625 mg/mL.
With S9 mix: 4.88, 9.77, 19.53, 39.06, 78.13, 156.25, 312.5 and 625 mg/mL.

Duplicate cultures were used for each treatment and two cultures were treated with the solvent control.

Positive control cultures were treated as in the first test.

Three hours after dosing, the cultures containing S9 mix were centrifuged. The cell pellets were rinsed and resuspended in fresh medium. They were then incubated for a further 18 hours. Cultures treated in the absence of S9 mix were incubated for 21 hours.

All cultures were treated with Colcemid, at a final concentration of 0.1 µg/mL, two hours before the end of the incubation period. They were then harvested, fixed and the slides prepared as previously described. The slides were then examined microscopically as previously described.

3. Assessment of results

An assay is considered to be acceptable if the negative and positive control values lie within the current historical control range.

The test substance is considered to cause a positive response if the following conditions are met:

- Statistically significant increases ($p<0.01$) in the frequency of metaphases with aberrant chromosomes (excluding gaps) are observed at one or more test concentration.
- The increases exceed the negative control range of this laboratory, taken at the 99 % confidence limit.
- The increases are reproducible between replicate cultures.
- The increases are not associated with large changes in osmolality of the treatment medium or extreme toxicity.
- Evidence of a dose-relationship is considered to support the conclusion.

A negative response is claimed if no statistically significant increases in the number of aberrant cells above concurrent control frequencies are observed, at any dose level.

A further evaluation may be carried out if the above criteria for a positive or a negative response are not met.

6. Statistics

Not mentioned, but under references: 'FISHER, R.A. (1973) The Exact Treatment of 2 x 2 Table in: Statistical Methods for Research Workers. Hafner Publishing Company, New York.' cited.

II. Results and Discussion

1. First test

In the absence of S9 mix, fluopicolide caused a reduction in the mitotic index to 53% of the solvent control value at 156.25 µg/mL. The dose levels selected for the metaphase analysis were 19.53, 78.13 and 156.25 µg/mL.

In the presence of S9 mix, fluopicolide caused a reduction in the mitotic index to 32% of the solvent control value at 625 µg/mL. The dose levels selected for the metaphase analysis were 78.13, 312.5 and 625 µg/mL.

The quantitative analysis for polyploidy showed no increase in the number of polyploid metaphase figures when compared to the solvent control.

The effects of fluopicolide on the chromosomes of cultured human lymphocytes are shown in the tables below.

In both the absence and the presence of S9 mix, fluopicolide caused no statistically significant increases in the proportion of cells with chromosomal aberrations at any dose level, when compared with the solvent control.

Both positive control compounds, mitomycin C and cyclophosphamide, caused large, statistically significant increases ($p < 0.001$) in the proportion of aberrant cells. This demonstrated the efficacy of the S9 mix and the sensitivity of the test system.

2. Second test

In the absence of S9 mix, fluopicolide caused a reduction in the mitotic index to 48% of the solvent control value at 19.53 µg/mL. The dose levels selected for the metaphase analysis were 1.22, 9.77 and 19.53 µg/mL.

In the presence of S9 mix, fluopicolide caused a reduction in the mitotic index to 50% of the solvent control value at 312.5 µg/mL. The dose levels selected for the metaphase analysis were 39.06, 156.25 and 312.5 µg/mL.

The quantitative analysis for polyploidy showed no increase in the number of polyploid metaphase cells when compared to the solvent control.

The effects of fluopicolide on the chromosomes of cultured human lymphocytes are shown in the following tables.

In both the absence and the presence of S9 mix, fluopicolide caused no statistically significant increases in the proportion of cells with chromosomal aberrations at any dose level, when compared with the solvent control.

Both positive control compounds, mitomycin C and cyclophosphamide, caused large, statistically significant increases ($p < 0.001$) in the proportion of aberrant cells.

Table 5.4.1- 19 and Table 5.4.1- 20 give an overview of the study results.

Table 5.4.1- 19: Relative mitotic indices and mean percentage of aberrant human lymphocytes, including and excluding gaps – Experiment I

Treatment	Concentration (µg/ml)	Treatment time (h)	Relative mitotic index percent (1000 cells)	Percent aberrant cells Including gaps	Percent aberrant cells Excluding gaps
Without metabolic activation (-S9)					
Fluopicolide	156.25	3	53	3.0	2.5
	78.13	3	71	2.5	1.5
	19.53	3	96	1.5	0.0
Solvent control	-	3	100	1.0	0.5
Positive control Mitomycin C	0.1	3	-	15.0***	8.5***
With metabolic activation (+S9)					
Fluopicolide	625	3	32	8.0	6.5
	312.5	3	63	5.5	5.0
	78.13	3	92	7.0	4.5
Solvent control	-	3	100	5.5	3.0
Positive control Cyclophosphamide	6	3	-	22.0***	18.5***

*** $p < 0.001$ statistically significantly different from controls Fisher's test

Table 5.4.1- 20: Relative mitotic indices and mean percentage of aberrant human lymphocytes, including and excluding gaps – Experiment II

Treatment	Concentration (µg/ml)	Treatment time (h)	Relative mitotic index percent. (1000 cells)	Percent aberrant cells Including gaps	Percent aberrant cells Excluding gaps
Without metabolic activation (-S9)					
Fluopicolide	19.53	21	48	2.0	0.5
	9.77	21	69	2.0	1.0
	1.22	21	100	1.5	0.5
Solvent control	-	21	100	1.0	0.0
Positive control Mitomycin C	0.1	21	-	19.5***	17.0***
With metabolic activation (+S9)					
Fluopicolide	312.50	3	50	2.0	1.0
	156.25	3	70	1.5	1.5
	39.06	3	90	1.5	1.0
Solvent control	-	3	700	2.0	1.5
Positive control Cyclophosphamide	6	3	-	18.5***	17.0***

*** p < 0.001 statistically signifcantly different from controls Fisher's test

III. Conclusion

It is concluded that fluopicolide has shown no evidence of clastogenic activity in this *in vitro* cytogenetic test system, under the experimental conditions described.

Assessment and conclusion by applicant:

The study was conducted according to OECD TG 473 and is valid and acceptable to assess the potential of fluopicolide to induce chromosome aberrations in human lymphocytes *in vitro*. Fluopicolide was not clastogenic under the conditions of this study.

Data Point:	KCA 5.4.1/08
Report Author:	[redacted]
Report Year:	2005
Report Title:	AE C638206: In vitro Chinese hamster lung V79 cell HPRT mutation test
Report No:	C026130
Document No:	M-210831-02-1
Guideline(s) followed in study:	OECD 476 (1997); US-EPA OPPTS 870.5300 (1998); EEC Directive 87/302 (1987)
Deviations from current test guideline:	Deviations from the current OECD guideline (476, 2016): None.
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

The study was performed to investigate the potential of fluopicolide (Batch No. PP/241024/2 & PP241067/1) to induce gene mutations at the HPRT locus in V 79 cells of the Chinese hamster *in vitro*.

The assay was performed in three independent experiments using identical procedures, both with and without rat liver microsomal activation (S9-mix). The test article was dissolved in DMSO and tested at the following concentrations:

First study: 3820.0*. 1208.0, 382.0, 120.8, 38.2, 12.1, 3.8 and 1.2 µg/mL

Second study: 120.0, 100.0, 75.0, 50.0, 25.0, 12.5, 6.3, 3.2, 1.6, 0.8 and 0.4 µg/mL

Third study: 60.0, 50.0, 40.0, 30.0, 20.0, 10.0, 5.0, 2.5, 1.25, 0.625 and 0.313 µg/mL

* = 10 mM, the international regulatory limit dose

The concentration ranges were based on the results of preliminary testing for solubility and toxicity. The highest concentration was toxic both with and without metabolic activation.

No relevant increase in mutant colony numbers was obtained in three independent experiments. The statistically significant increase observed in the presence of S9-mix was caused by a low mutation frequency of the solvent control values and therefore this increase was considered to be without any relevance. In the second main study a statistically significant increase in the number of mutant colonies was observed at the concentrations of 0.8 and 12.5 µg/mL without metabolic activation. This was considered to be without biological relevance because at the dose of 12.5 µg/mL it was not three-fold higher than the solvent control value, no dose-dependency was obtained and the findings were not reproduced in the third experiment.

The positive controls showed distinct statistically significant increases in induced mutant colonies, thus indicating the sensitivity of the assay.

Based on these results, fluopicolide was not mutagenic in this HPRT test with V79 Chinese hamster cells.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 (fluopicolide)
Purity:	97.8% (w/w)
Batch no.:	Mixture of PP/241024/2 & PP241067/1

2. Vehicle and/or positive control

Vehicle:	DMSO
Positive control:	Non-activation: Ethyl methane sulfonate (EMS) Activation: 9,10-dimethyl-1,2-benzanthracene (DMBA)

3. Activation:

The S9 fraction was prepared by the department conducting the study according to Ames *et al.* (1975)[13]. Male Sprague Dawley rats (200-300 g), supplied by Harlan Winkelmann (33178 Borchen, Germany), received a single intraperitoneal injection of Aroclor 1254 (500 mg/kg bw) five days before killing.

The livers were removed from at least 5-6 animals using cold sterile solutions at approx. 0 to 4 °C and glassware, and were then pooled and washed in approx. 150 mM KCl (approx. 1 mL/g wet liver). The washed livers were cut into small pieces and homogenized in three volumes of KCl. The homogenate was centrifuged at approx. 9000 x g for 10 minutes. The supernatant is the S9 fraction. This was divided into small portions, rapidly frozen and stored at approx. -80 °C for not longer than six months.

The protein content was determined for every batch. Also for every batch of S9 an independent validation was performed with a minimum of two different mutagens, e.g. 2-aminoanthracene and dimethylbenzanthracene, to confirm metabolic activation by microsomal enzymes.

The preparation of S9-mix was s follows. Sufficient S9 fraction was thawed immediately at room temperature before each test. An appropriate quantity of S9 fraction (batch no. 99/6 for all experiments, protein concentration 55.0 g/L) was mixed with S9 cofactor solution to yield a final protein concentration of 0.3 mg/mL in the cultures which was kept on ice until used. This preparation is termed S9-mix.

The concentrations of the different compounds in the S9-mix were:

8 mM $MgCl_2$
33 mM KCl
5 mM glucose-6-phosphate
5 mM NADP
100 mM phosphate buffer pH 7.4

4. Cell cultures and medium:

Large stocks of the mycoplasma-free V79 cell line are stored in liquid nitrogen in the cell bank of "Genetic Toxicology", thus permitting repeated use of the same cell culture batch for numerous experiments. The identical characteristics of the cells ensure comparability of the experimental parameters.

Thawed stock cultures are kept at approx. 37 °C and approx. 4% CO_2 in 175 cm^2 plastic flasks. About $5x10^5$ to $1x10^6$ cells were seeded into each flask in 30 mL of MEM-medium supplement with approx. 10% (v/v) FCS (foetal calf serum) containing approx. 2 mM L-glutamine and approx. 0.1% (w/v) neomycinsulfate. The cells were sub-cultured twice a week. For the selection of mutants the medium was supplemented with approx. 11 µg/mL thioguanine.

5. Test substance concentrations used:

The following test substance concentrations were used:

Table 5.4.1- 21: Test substance concentrations

Experiment	S9 mix	Concentration in culture medium (µg/mL)
1st Experiment	+/-	1.2, 3.8, 12.1, 38.2, 120.8*, 382.0*, 1208.0*, 3820.0*
2nd Experiment	+/-	0.4, 0.8, 1.6, 3.2, 6.3, 12.5, 25.0, 50.0**, 75.0, 100.0*, 120.0*
3rd Experiment	+/-	0.313, 0.625, 1.25, 2.5, 5.0, 10.0, 20.0, 30.0, 40.0**, 50.0**, 60*

* because of high toxicity no mutant selection was performed with and without S9 mix
** because of high toxicity no mutant selection was performed with S9 mix

B. Test performance

Experimental phase: April 4 to May 19, 2000

1. Preliminary assay

A preliminary toxicity test was undertaken in order to select appropriate dose levels for the mutation assay. In the test a wide range of dose levels of test substance was used. Cell cultures were subjected to the same treatment conditions as in mutation assays, and the survival of the cells was subsequently determined.

The test included the following treatments:

Solvent control: The maximum final concentration of organic solvents will not exceed approx. 1% (v/v).

Test substance: The highest dose level for the preliminary cytotoxicity test was determined by the solubility of the test substance up to the maximum of 10 mM or 5000 µg/mL.

Treatments were performed both in the presence and absence of S9 metabolic activation system using a single cell culture.

2. Main assay

In preliminary toxicity experiments approximately 4500 cells were seeded in each well of a microtiter plate, allowed to attach overnight and exposed to the test and control compound for four hours.

For each concentration at least 6 wells were used. Approximately 24 hours after treatment, the cells were fixed and stained with crystal violet. Survival was determined by measurement of the crystal violet extinction.

In the main experiments the cultures were prepared and treated with the test substance in the same way as for the preliminary experiment. 24 hours after seeding of approx. 4500 cells per well in a microtiter plate, the medium was replaced with serum-reduced (5% v/v) medium containing the test substance, either without S9-mix or with S9-mix. After 4 hours the treatment medium was removed and the cells were rinsed twice with normal medium. Thereafter normal medium was added to the wells. The cultures were stained with crystal violet and survival was determined after an incubation period of approx. 24 hours.

As rationale for dose selection, for non-toxic, freely soluble test substances, the top dose is 10 mM or 5000 µg/mL according to international testing guidelines. For non-toxic, poorly soluble test substances, the top dose is the highest evaluable dose. For toxic compounds a percentage survival rate relative to the solvent control was calculated for each treatment. The dose level which results in a predicted survival of about 30% was estimated from the results obtained. This dose was chosen as the highest dose level. At least eleven single dose levels or four duplicated doses are included in the treatment series.

Three independent mutation tests were performed.

Exponentially growing cultures which were more than 50% confluent were trypsinated by an approx. 0.25% (v/v) trypsin ready for use (mfr. Gibco). A single cell suspension was prepared. Subsequently the cells were replated to determine the mutation frequency and plating efficiency.

The treatment schedule of the mutagenicity test is described below:

Day 1: Sub-culturing of an exponentially growing culture

a) Approx. 4500 cells in each well of a microtiter plate for determination of the plating efficiency.

b) $6x10^5 – 1x10^6$ cells in 175 cm^2 flasks with 30 mL medium for the mutagenicity test, one flask per experimental point.

Day 2: Treatment of a) and b) with the test substance in the presence and absence of S9-mix (final protein concentration approx. 0.3 mg/mL) for 4 hours.

Day 3: Fixation and staining of the cells in a) microtiter plate for the determination of the plating efficiency.

Day 5 or 6: Subculturing of b) in 175 cm^2 flasks.

Day 9: Subculturing of b) in five 75 cm^2 flasks with culture medium containing 6-thioguanine: Mutant selection (about 300 000 cells/flask); Subculturing of b) in two 25 cm^2 flasks for plating efficiency (about 400 cells per flask).

Day 16: Fixation and staining of colonies of b) – from subcultures seeded on day 9.

All incubations were carried out at approx. 37 °C and 4 % CO_2. Staining was performed with approx. 10% (v/v) methylene blue in approx. 0.01% KOH solution. Only colonies with more than 50 cells were counted.

3. Acceptance Criteria

This assay was considered valid if the following criteria were met:

- The spontaneous control data were within the laboratory's normal control range for the spontaneous mutant frequency.
- The positive controls induced increases in the mutation frequency which were both statistically significant and within the laboratory's normal range.
- The plating efficacy for the solvent control was greater than 50%.

4. Criteria for a positive response

The test substance is classified as mutagenic if:

- It reproducibly induces with one of the test compound concentrations a mutation frequency that is three times higher than the spontaneous mutant frequency in this experiment.
- There is a reproducible concentration-related increase in the mutation frequency. Such an evaluation may be considered independently from the enhancement factor for induced mutants.
- Survival of the responding dose group is at least 30%.

However, in a case by case evaluation both decisions depend on the level of the corresponding negative control data.

5. Statistical analysis

The biometry of the results was performed off-line with the Mann-Whitney-U-Test.

II. Results and Discussion

A. Preliminary cytotoxicity assay

Fluopicolide was suspended in DMSO. Evaluation of the solubility of that suspension in cell culture medium showed that 3820 µg/mL was the highest practicable concentration and produced a heavy precipitate. This concentration corresponds to 10 mM, which is the highest dose level tolerated to be tested and the recommended international regulatory limit dose.

Accordingly, the preliminary toxicity study was carried out in microtiter plates using a maximum concentration of 3820 µg/mL and a wide range of lower dose levels down to 10 µg/mL.

Following treatment in the absence of S9 metabolic activation, high toxicity was observed. Survival declined in a dose-related manner reaching 36.9% of the solvent control value at the highest dose level. In the presence of S9-mix a broadly similar response was seen. Survival reached 56.3% of the solvent control value at the highest dose level. Macroscopic precipitation of the test substance in the medium was observed at 250 µg/mL and above, whilst microscopic precipitation was obtained at 50 µg/mL and higher.

On the basis of these results, a concentration of 3820 µg/mL was used for the first main assay and seven lower dose levels down to 1.2 µg/mL were included in the treatment series.

B. Main assay

Plating efficiency (microtiter plates)

In the absence of S9 metabolic activation in all mutation experiments a dose-related decrease in survival was observed in the microtiter plates. In the first assay survival declined reaching 45.9% of the solvent control value at the highest dose level (3820 µg/mL). In the second mutation test survival was reduced to 74.4% at the concentration of 120 µg/mL, in the third experiment survival declined to 56.7% at the highest dose level of 60 µg/mL. In the presence of S9 metabolic activation survival in microtiter plates also decreased in a dose-related manner reaching 44.5% in the first study at a dose level of 3820 µg/mL. In the second and third experiment survival declined reaching 57.4%, and 62.6% of the solvent control value in the microtiter plates at 120.0 µg/mL and 60.0 µg/mL, respectively.

Macroscopic precipitation of the test substance in the medium was observed at 382.0 µg/mL and above, whilst microscopic precipitation was obtained at 30 µg/mL and higher.

In all assays survival in microtiter plates, which were stained 24 hours after treatment, was not comparable with the mass cultures, which were subcultured five days after treatment.

Mutagenicity test (mass culture)

In the first main experiment marked decrease in the mass culture was observed at the concentration of 120.8 µg/mL with and without S9-mix, where survival was reduced to 2.9% and 2.5% of the solvent control value, respectively. Therefore, the mutation selection was possible only up to a dose level of 38.2 µg/mL .

Based on these results the second main study was carried out using a maximum concentration of 120 µg/mL.

In the second main study survival in the mass culture was markedly decreased to 1.5% of the solvent control value at the concentration of 75 µg/ml without S9-mix. In the presence of S9 metabolic activation high toxicity was also observed, reaching 4.3% at the dose level of 50 µg/mL. Therefore, the mutation selection could be performed only up to dose levels of 50 µg/mL (without metabolic activation) and 25 µg/mL (with metabolic activation).

Based on these results the third main study was carried out using a maximum concentration of 60 µg/mL.

In the third mutation assay without S9-mix, survival in mass culture was reduced to 1.6% of the solvent control value at the highest concentration (60 µg/mL). In the presence of a metabolic activation high toxicity was obtained reaching 1.4% of the solvent control value at the dose level of 40 µg/mL. Therefore, the mutation selection was possible only up to a dose level of 50 µg/mL (without metabolic activation) and 30 µg/mL (with metabolic activation).

Fluopicolide was assessed for its mutagenic potential in vitro in this HPRT-test in three independent experiments without metabolic activation and three independent experiments with metabolic activation. The results of these experiments are presented in the following tables.

No relevant reproducible increase of the mutant colonies or mutant frequency over the range of the solvent control was found with any of the concentrations used with metabolic activation by S9-mix.

The statistically significant increase observed in the first main study in the presence of S9-mix was caused by a low mutation frequency of the solvent control values and therefore were without any relevance. In the second main assay, there was a statistically significant increase of the mutation rate over the range of the solvent controls at 0.8 and 12.5 µg/mL without metabolic activation. These findings were without biological relevance because at the dose level of 12.5 µg/mL the increase was not three-fold higher than the solvent control value, no dose related response was observed and the results were not reproduced in the third experiment.

The sensitivity of the test system was demonstrated by the enhanced mutation frequency in the cell cultures treated with the positive control compounds.

Table 5.4.1- 22: Relative survival and mean mutation frequency (mutant colonies per 1 millions cells) – Experiment I

Treatment	Concentration (µg/ml)	Relative survival (%)	Mutation frequency
Without metabolic activation (-S9)			
Fluopicolide	3820.0	2.5	NA
	1208.0	1.7	NA
	382.0	2.1	NA
	120.8	2.9	NA
	38.2	129.5	12.6
	12.1	144.0	15.7
	3.8	120.3	14.5
	1.2	99.6	13.5
Negative control	-	100.0	6.5
Solvent control	-	100.0	17.1
Positive control EMS	1000	90.9	742.6*
With metabolic activation (+S9)			
Fluopicolide	3820.0	2.5	NA
	1208.0	1.7	NA
	382.0	2.1	NA
	120.8	2.9	NA
	38.2	129.5	12.8
	12.1	144.0	14.2
	3.8	120.3	25.1*
	1.2	99.6	23.7*
Negative control	-	100.0	21.4
Solvent control	-	100.0	11.2
Positive control DMBA	7.7	74.8	128.4*

NA: not applicable as % relative survival < 50 %
*: $p < 0.05$ statistically significant Mann-Whitney-U-Test

Table 5.4.1- 23: Relative survival and mean mutation frequency (mutant colonies per 1 millions cells) –Experiment II

Treatment	Concentration (µg/ml)	Relative survival (%)	Mutation frequency
Without metabolic activation (-S9)			
Fluopicolide	120.0	2.2	NA
	100.0	1.5	NA
	75.0	1.5	NA
	50.0	108.2	11.4
	25.0	121.7	11.1
	12.5	106.7	26.2*
	6.3	104.9	19.8
	3.2	113.1	21.2
	1.6	98.5	9.7
	0.8	98.9	15.9*
	0.4	115.0	17.2
Negative control	-	100.0	13.2
Solvent control	-	100.0	13.3
Positive control EMS	1000	70.0	1025.2*
With metabolic activation (+S9)			
Fluopicolide	120.0	2.4	NA
	100.0	1.9	NA
	75.0	1.9	NA
	50.0	4.3	NA
	25.0	118.4	31.6
	12.5	143.5	12.8
	6.3	155.1	24.3
	3.2	121.3	20.5
	1.6	108.4	12.3
	0.8	157.0	20.4
	0.4	163.3	23.8
Negative control	-	100.0	18.1
Solvent control	-	100.0	27.7
Positive control DMBA	7.7	86.0	169.2*

NA: not applicable as % relative survival < 50 %
*: p<0.05 statistically significant Mann-Whitney-U-Test

Table 5.4.1- 24: Relative survival and mean mutation frequency (mutant colonies per 1 millions cells) – Experiment III

Treatment	Concentration (µg/ml)	Relative survival (%)	Mutation frequency
Without metabolic activation (-S9)			
Fluopicolide	60.0	1.6	NA
	50.0	65.5	19.0
	40.0	108.1	25.1
	30.0	117.1	14.9
	20.0	146.0	22.7
	10.0	102.5	9.0
	5.0	113.7	11.8
	2.5	112.4	18.9
	1.25	89.1	30.8
	0.625	141.9	7.0
	0.313	134.8	31.6
Negative control	-	100.0	12.1
Solvent control	-	100.0	16.9
Positive control EMS	1000	113.7	889.1*
With metabolic activation (+S9)			
Fluopicolide	60.0	0.8	NA
	50.0	0.8	NA
	40.0	1.4	NA
	30.0	104.2	17.7
	20.0	106.4	27.0
	10.0	99.7	20.4
	5.0	111.7	10.0
	2.5	105.3	27.5
	1.25	108.4	27.6
	0.625	112.8	10.9
	0.313	95.5	15.4
Negative control	-	100.0	12.0
Solvent control	-	100.0	20.3
Positive control DMBA	7.7	96.7	93.3*

NA: not applicable as % relative survival < 50 %
*: $p<0.05$ statistically significant Mann-Whitney-U-Test

III. Conclusion

Fluopicolide did not induce gene mutation in either the presence or absence of metabolic activation, i.e. was not mutagenic, in this HPRT test with V79 Chinese hamster cells.

Assessment and conclusion by applicant:

The study was conducted according to OECD 476 and is valid and acceptable to assess the potential of fluopicolide to induce gene mutations at the HPRT locus in Chines Hamster Lung V79 cells. Fluopicolide is not mutagenic in mammalian cells under the conditions of this study.

CA 5.4.2 In vivo studies in somatic cells

Data Point:	KCA 5.4.2/01
Report Author:	[redacted]
Report Year:	2005
Report Title:	AE C638206 00 1C99 0005 - Mouse erythrocyte micronucleus test
Report No:	C008175
Document No:	M-197261-02-1
Guideline(s) followed in study:	OECD 474 (1997); US-EPA OPPTS 870.5395 (1998); EU Directive 92/69/EEC Annex B.12.
Deviations from current test guideline:	Deviations from the current OECD guideline (474, 2016): - Exposure of the target organ, i.e. bone marrow, was not verified - 2000 instead of the currently required 4000 immature erythrocytes were analysed - For the assessment of the ratio of polychromatic to total erythrocytes, 200 instead of the required 500 erythrocytes were analysed. However, bone marrow exposure was shown in an additional micronucleus test in vivo with i.p. administration ([redacted], 2003; M-223119-01-1) which showed no indication of a clastogenic effect of fluopicolide. Therefore, the results of the present study are acceptable as supplementary information.
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

In this study the potential of fluopicolide to induce chromosomal damage (clastogenicity) in mouse bone marrow erythrocytes as evidenced by micronuclei formation was assessed.

Groups of five male and five female NMRI mice were used. They were 7 weeks of age and had mean body weights of 33.6 g (males) and 26.4 g (females) at the start of the study (first dose). Each mouse was given two gavage doses separated by an interval of 24 hours of either 200, 600 or 2000 mg/kg bw of fluopicolide in aqueous methylcellulose (1% w/v). The highest dose level was the international regulatory limit dose. The animals were killed 24 hours after the second dose.

Cyclophosphamide (Endoxan) the positive control substance, was administered as a single oral (gavage) dose of 50 mg/kg bw to five male and five female mice which were killed 24 hours after dosing.

Bone marrow smears were prepared from each animal and one from each was examined for the presence of micronuclei in 2000 polychromatic erythrocytes. The ratio of polychromatic to 200 erythrocytes was assessed.

Treatment with fluopicolide did not increase the number of polychromatic erythrocytes containing micronuclei. The ratio of polychromatic to normochromatic erythrocytes was not significantly affected.

Thus, fluopicolide was not clastogenic in this mouse erythrocyte micronucleus test.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 (fluopicolide)
Purity:	97.8% (w/w)
Batch no.:	Mixture of PP124102412 & PPl24106711

2. Vehicle and/or positive control

Vehicle:	Aqueous methylcellulose 1 % (w/v)
Positive control:	Cyclophosphamide

3. Test animals

Species:	Mouse
Strain:	HsdWin:NMRI
Age:	Approx. 7 weeks
Weight at start:	30-36 g (males), 23-30 g (females)
Source:	[redacted]
Acclimation period:	Yes
Diet:	Rat/mouse diet ssniff RM-H (V 1534), ad libitum ssniff GmbH, Postbox 2039, 59480 Soest
Water:	Potable water taken from the public supply was freely available via polycarbonate bottles fitted with sipper tubes, except when urine was being collected
Housing:	Fully air-conditioned rooms in makrolon cages type 4 (five animals of the same sex per cage) on soft wood granulate
Temperature:	22 ± 3 °C
Humidity:	50 ± 20%
Air changes:	Fully air-conditioned
Photoperiod:	12 hours

4. Test substance doses

Gavage administrations of 200, 600, 2000 mg/kg bw, in two doses, separated by 24 hours.

B. Test performance

Experimental phase: April 3 to April 28, 2000

1. Treatment and sampling times

The study design of the main study was as follows.

Table 5.4.2- 1: Micronucleus test design

Experimental group	Dose in mg/kg bw	No. of animals per sex	Route and number of applications	
Negative control	0	5	oral	2
Fluopicolide	200	5	oral	2
	600	5	oral	2
	2000	5	oral	2
Positive control (Cyclophosphamide)	50	5	oral	1

The test substance was administered two doses separated by an interval of 24 hours orally by gavage to the test animals at doses of 200, 600 and 2000 mg/kg bw. The vehicle, aqueous methylcellulose 1 % (w/v), was administered in the same way to the negative control groups. The study included a concurrent positive control using cyclophosphamide (Endoxan) which was administered once orally by gavage at a dose of 50 mg/kg bw.

Following dosing, the animals were examined regularly for mortality and clinical signs of toxicity.

2. Tissues and cells examined

For the extraction of the bone marrow, the animals were killed by carbon dioxide asphyxiation 24 hours after the last dose. Two femora were removed and the bones freed of muscle tissue. The proximal ends of the femora were opened, the bone marrow flushed into a centrifuge tube containing approx. 3 mL of foetal bovine serum and a suspension is prepared. The mixture was then centrifuged for 5 minutes at approx. 1200 rpm, after which almost all the supernatant was discarded. One drop of the thoroughly mixed sediment was smeared onto a cleaned slide, identified by project code and animal number and air-dried for approx. 12 hours.

Subsequently the slides are stained as follows:

- 5 minutes in methanol
- 5 minutes in May-Grünwald's solution brief rinsing twice in distilled water
- 10 minutes staining in 1 part Giemsa solution to 6 parts buffer solution, pH 7.2 (Weise)
- rinsing in distilled water
- drying
- coating with Entellan.

3. Scoring

2000 polychromatic erythrocytes were counted for each animal. The number of cells with micronuclei was recorded, not the number of individual micronuclei. In addition, the ratio of polychromatic erythrocytes to 200 erythrocytes was determined. Main parameter for the statistical analysis, i.e. validity assessment of the study and mutagenicity of the test substance, was the proportion of polychromatic erythrocytes with micronuclei out of the 2000 counted erythrocytes. All bone marrow smears for evaluation were coded to ensure that the group from which they were taken remained unknown to the investigator.

4. Evaluation criteria

A one-sided Wilcoxon-Test (Hollander, M.; Wolfe, D.A.: Nonparametric statistical methods; Wiley Seriesin Probability and Mathematical Statistics; John Wiley & Sons, Inc.; New York (1973) Streitberg, B.; Rdhmel, J.: Exakte Verteilung fur Rang- und Randornisierungstests irn allgemeinen c-Stichprobenproblem; EDV in Medizin und Biologie 18; 12 - 19; Verlag Eugen Ulmer GmbH & Co., Stuttgart; Gustav Fischer Verlag KG, Stuttgart (1987) was used to check the validity of the study. The study was considered as valid if the proportion of polychromatic erythrocytes with micronuclei in the positive control was significantly higher than in the negative control ($p = 0.05$).

5. Criteria for a positive response

Both biological and statistical significances were considered together for evaluation purposes.

A substance is considered as positive if there is a significant dose-related increase in the number of micronucleated polychromatic erythrocytes compared with the concurrent negative control group. A test substance producing no significant dose-related increase in the number of micronucleated polychromatic erythrocytes is considered non-mutagenic in this system.

6. Statistical methods

Assuming the study is valid based on a monotone dose-relationship, one-sided Wilcoxon tests were performed initially comparing control values with those of the highest dose group. Tests on lower dose groups were only performed if all higher dose groups were significantly different from the control[17]. A significance level of 5% is adopted for all tests.

II. Results and Discussion

A. Micronucleus assay

Mice were given 2 doses of 200, 600 and 2000 mg fluopicolide per kg body weight separated by an interval of 24 hours to study the induction of micronuclei in bone marrow cells.

All animals survived after treatment. No signs of toxicity were observed. The dissection of the animals revealed no test substance related macroscopic findings.

The bone marrow smears were examined for the occurrence of micronuclei in red blood cells. The results are summarized in Table 5.4.2-2.

The incidence of micronucleated polychromatic erythrocytes in the dose groups of fluopicolide was within the normal range of the negative control groups. No statistically significant increase of micronucleated polychromatic erythrocytes was observed.

The ratio of polychromatic erythrocytes to total erythrocytes (PCE/Ery ratio) remained essentially unaffected by the test compound and was not less than 20% of the control values.

Cyclophosphamide (Endoxan B) induced a marked and statistically significant increase in the number of polychromatic erythrocytes with micronuclei, thus indicating the sensitivity of the test system.

An overview is given in Table 5.4.2- 2.

[17] Hothom, L.; Lehmacher, W.: A Simple Testing Procedure "Control versus k Treatments" for One-sided Ordered Alternatives, with Application in Toxicology; Biom. J. 33,179 - 189; Akademie Verlag (1991)

Table 5.4.2- 2: Group mean PCE/NCE ratios and incidences of micronucleated PCE

Treatment	Dose (mg/kg bw)	No. of animals	Total no. PCE scored	PCE/Ery ratio ± SD	Mean number micronucleated PCE ± SD
Males					
Fluopicolide	2000	5	10000	0.47 ± 0.06	1.6 ± 0.04
	600	5	10000	0.45 ± 0.02	1.6 ± 0.04
	200	5	10000	0.44 ± 0.06	1.8 ± 004
	0	5	10000	0.48 ± 0.07	1.0 ± 0.04
Positive control (Cyclophosphamide)	50	5	10000	0.46 ± 0.04	72.2 ± 1.03
Females					
Positive control (Cyclophosphamide)	2000	5	10000	0.48 ± 0.05	1.0 ± 0.04
	600	5	10000	0.46 ± 0.06	2.0 ± 0.04
	200	5	10000	0.53 ± 0.03	1.8 ± 0.07
	0	5	10000	0.47 ± 0.06	1.6 ± 0.04
Positive control (Cyclophosphamide)	50	5	10000	0.55 ± 0.03	55.8 ± 1.13

PCE: polychromatic erythrocytes
NCE: normochromatic erythrocytes
Ery: total erythrocytes (PCE+NCE)

III. Conclusion

Fluopicolide did not induce clastogenicity. It was not mutagenic in this micronucleus test.

Assessment and conclusion by applicant:

The study was conducted in accordance with OECD TG 474 and is acceptable to assess the clastogenic potential of fluopicolide with the caveat of the deviations described. Bone marrow exposure was not demonstrated; however, exposure was shown in an additional micronucleus test *in vivo* with i.p. administration ([redacted] 2003; M-223119-01-1) which showed no indication of a clastogenic effect of fluopicolide. Therefore, the results of the present study are acceptable as supplementary information.

Data Point:	KCA 5.4.2/02
Report Author:	[redacted]
Report Year:	2003
Report Title:	AE C638206: Induction of micronuclei in the bone marrow of treated mice
Report No:	C035885
Document No:	M-219364-01-1
Guideline(s) followed in study:	OECD: 474 (1997); ICH Tripartite Harmonised Guideline on Genotoxicity (1995); US-EPA OPPTS 870.5395 (1998)
Deviations from current test guideline:	Method: Deviations from current guideline SANCO/3029/99 rev. 4: There is no calibration plot or calibration equation presented, however the calibration range and correlation coefficient (0.9999) are reported. For the accuracy and precision data, there is only 1 concentration level prepared in matrix (LOQ samples). However, there are 6 determinations at this level with a mean recovery between 70-110% and RSD <20%. Considering that this analytical method is validated in support of a toxicological cold study, the method validation is considered fit for purpose. Study: Deviations from the current OECD guideline (474, 2016): - Exposure of the target organ, i.e. bone marrow, was not verified. However, bone marrow exposure was shown in an additional micronucleus test in vivo ([redacted] 2003; M-223119-01-1) which showed no indication of a clastogenic effect of fluopicolide. Therefore, the results of the present study are acceptable as supplementary information.
Previous evaluation:	yes, evaluated and accepted in DAR 2005
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Supportive only

Executive Summary:

Fluopicolide was assayed *in vivo* in a mouse bone marrow micronucleus test at a single limit dose.

For the range-finder experiment, fluopicolide was formulated in 1% (w/v) aqueous methylcellulose (1% MC) and administered once daily on two consecutive days to a group of three male mice at a dose of 2000 mg/kg bw/day (the recommended maximum dose level for *in vivo* cytogenetic studies according to current regulatory guidelines).

Observations were made over a two-day period following the second administration and signs of toxicity recorded. As no clinical signs of toxicity were observed, so the main experiment was conducted using at a single limit concentration (2000 mg/kg bw/day).

In the main study, fluopicolide was formulated as described and administered at 2000 mg/kg bw/day to a group of six male mice killed 24 hours after the second administration.

The negative (vehicle) control in the study was 1% MC also administered orally by gavage once daily on two consecutive days to a group of six male mice. These animals were killed 24 hours after the second administration.

Cyclophosphamide (CPA), the positive control, was dissolved in saline and administered orally by gavage as a single dose of 40 mg/kg bw to a group of six male mice which were killed after 24 hours. Positive control animals exhibited increased numbers of micronucleated polychromatic erythrocytes (PCE) such that the micronucleus frequency in the positive control group was significantly greater than in concurrent controls.

Negative (vehicle) control mice exhibited a group mean ratio of PCE to NCE (normochromatic erythrocytes) which was within the historical negative control range. However, it was noted that one animal showed a high number of micronucleated PCE (2.25 micronucleated PCE/1000 cells) that clearly exceeds the historical negative control data frequency (0.40 micronucleated PCE/1000 cells).

However, this was observed in just one animal in a total of six and all other vehicle control animals exhibited numbers of micronucleated PCE that were similar to the expected distribution. The vehicle control data was therefore considered valid.

Mice treated with fluopicolide at 2000 mg/kg bw/day exhibited a group mean ratio of PCE to NCE which was similar to the value for the vehicle control group and which lay within the historical control data range. The group mean frequency of micronucleated PCE in the test substance treated group was slightly increased compared to the concurrent negative control group value (1.50 ± 0.8 and 0.88 ± 0.70 respectively), but without any statistical significance. As such the protocol criteria for a positive result were not met. This is supported by the fact that an incidentally high number of MN PCE (9 MN PCE per 4000 analysed) was also detected in one control animal (see above) and the group mean value of the positive control was significantly higher than the mean value of fluopicolide treatment group.

It is concluded that treatment of mice with fluopicolide at 2000 mg/kg bw/day (the recommended maximum dose level for *in vivo* cytogenetic studies according to current regulatory guidelines) resulted in a group mean frequency of micronucleated PCE in the test group that was slightly increased compared to the concurrent negative control group value. This result was statistically non-significant and of questionable biological significance.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 (fluopicolide)
Purity:	96.1% (w/w)
Batch no.:	OP 2050046

2. Vehicle and/or positive control

Vehicle:	Aqueous methylcellulose 1% (w/v)
Positive control:	Cyclophosphamide

3. Test animals

Species:	Mouse
Strain:	Male out bred CD-1 Crl:CD-l™(ICR) BR mice
Age:	4-6 weeks
Weight at start:	22-32 g
Source:	[redacted]
Acclimation period:	Yes
Diet:	Special Diets Services Ltd, RM1.(E).SQC.)
Water:	Bottled water (public supply)
Housing:	Groups of no more than three animals in solid-floored cages, cleaned and dried before use with wood shavings for bedding.
Temperature:	19.7-21.5 °C
Humidity:	40-70%
Air changes:	At least 15/hour
Photoperiod:	12 hours

4. Test substance doses

Gavage administrations of 2000 mg/kg bw, in two doses, separated by 24 hours (based on a range finding study).

B. Test performance

Experimental phase: March 20 to June 12, 2003

1. Treatment and sampling times

The study design of the main study was as follows.

Table 5.4.2- 3: Micronucleus test design

Experimental group	Dose in mg/kg bw	No. of animals	Route and number of applications	
Range finding study				
Fluopicolide	2000	3	oral	2
Main study				
Negative control	0	6	oral	2
Fluopicolide	2000	6	oral	2
Positive control (Cyclophosphamide)	40	6	oral	1

Animals were dosed once daily for two consecutive days with the test substance or vehicle. The positive control was given as a single administration at 40 mg/kg bw, on the second day of dosing (24 hours prior to harvest).

2. Tissues and cells examined

Test substance and vehicle treated mice were killed in groups, 24 hours after the second administration; CPA-treated mice were killed 24 hours after the single dose. Mice were killed by asphyxiation with carbon dioxide (subsequently ensured by cervical dislocation) in the same order as they were dosed.

Both femurs from each animal were exposed, removed, cleaned of adherent tissue and the ends removed from the shanks. Using a syringe and needle, bone marrows were flushed from the marrow cavity with 1 mL foetal bovine serum into appropriately labelled centrifuge tubes (one per animal).

The tubes were centrifuged (1250 x *g*, 2-3 minutes) and the serum was aspirated to leave one or two drops and the cell pellet. The pellet was mixed into this small volume of serum in each tube and from each tube a small volume of suspension was placed on the end of each of two slides labelled with the appropriate study number, sampling time, sex, date of preparation and animal number. The latter served as a code so analysis could be conducted "blind". A smear was made from the drop by drawing the end of a clean slide along the labelled slide.

Slides were allowed to air dry and were fixed for 5 minutes in absolute methanol, followed by rinsing several times in water. One slide from each set of two was then taken, the other was kept in reserve. After a second fixing/rinsing procedure, slides were stained according to the modification of Gollapudi and Kamra[18]. Slides were stained for 10 minutes in filtered Giemsa stain diluted 1:6 (v/v) in distilled

[18] G. Gollapudi B. and Kamra O.P. (1979): Application of a simple Giemsa staining method in the micronucleus test. Mutation Res 64, 45-46

water. Stained slides were rinsed, and allowed to dry thoroughly before clearing in xylene for 3 minutes. When dry, the slides were mounted with coverslips.

In this study, a reserve set of slides were stained (as detailed above) and analysed in order to generate additional data.

3. Scoring

Slides from the CPA-treated mice were initially checked at Covance Laboratories Limited to ensure the system was operating satisfactorily. The slides from all control and dose groups were arranged in numerical order by sampling time and analysed by a person not connected with the dosing phase of the study. Initially the relative proportions of polychromatic erythrocytes (PCE), seen as pale blue or blue/grey enucleate cells, and normochromatic erythrocytes (NCE), seen as smaller yellow/orange-stained enucleate cells, were determined until a total of at least 1000 cells (PCE plus NCE) had been analysed. Counting continued (but of PCE only) until at least 2000 PCE per animal had been observed (where possible). All PCE containing micronuclei observed during these two phases of counting were recorded. The vernier coordinates of all cells containing micronuclei were recorded to a maximum of six per 2000 cells scored. In order to obtain more data for each animal additional scoring was performed to provide a total of 2000 PCE + NCE and a total of 4000 PCE per animal had been examined for micronuclei (where possible).

Slide analysis was performed by an analyst trained in accordance with Covance Laboratories Limited Standard Operating Procedures. Details of the analyst are included in the responsible personnel list. All slides and raw data have been retained at Covance Laboratories Limited for archiving in accordance with the archive statement in this report.

4. Evaluation criteria

After completion of microscopic analysis and decoding of the data, the ratio of PCE/NCE for each animal and the mean for each group was calculated. The individual and group mean frequency of micronucleated PCE/1000 cells (+ standard deviation) were also determined.

PCE/NCE ratios were examined to see if there was any decrease in groups of treated animals that could be taken as evidence of bone marrow toxicity. The group mean frequencies of micronucleated PCE in vehicle control animals were compared with the historical negative control range to determine whether or not the assay was acceptable.

5. Acceptance criteria

The assay is considered valid if the following criteria are met:

1. The incidence of micronucleated PCE in the vehicle control group falls within or close to the historical vehicle control range, and
2. At least five animals (males) out of each group are available for analysis, and
3. The positive control chemical (CPA) induced a statistically significant increase in the frequency of micronucleated PCE.

6. Criteria for a positive response

A test substance is considered as positive in this assay if:

1. A statistically significant increase in the frequency of micronucleated PCE occurs at least at one dose, and
2. The frequency of micronucleated PCE at such a point exceeds the historical vehicle control range.

7. Statistical methods

For each group, inter-individual variation in the numbers of micronucleated PCE was estimated by means of a heterogeneity chi-square test.

The numbers of micronucleated PCE in each treated group were then compared with the numbers in vehicle control groups by using a 2 x 2 contingency table to determine chi-square. Probability values of $p < 0.05$ were to be accepted as significant. A further statistical test (for linear trend) was used to evaluate possible dose-response relationships.

If the heterogeneity chi-square test provides evidence of significant ($p < 0.05$) variability between animals within at least one group, non-parametric analysis is more appropriate. Provision was made to use the Wilcoxon rank sum test under these circumstances.

In this study, small, but statistically significant heterogeneity was observed within the vehicle control group and as such additional statistical analysis using non parametric analysis was considered appropriate. The Wilcoxon rank sum test was performed to compare the vehicle against the test article treated group.

II. Results and Discussion

A. Micronucleus assay

As no clinical signs of toxicity were observed in the range finding study, a single limit dose of 2000 mg/kg bw/day (the recommended maximum dose for *in vivo* cytogenetic assays, according to current regulatory guidelines) was chosen for the main study.

In the main study, no clinical signs of toxicity were observed in any treated animal.

The results of the formulations analysis indicated that the achieved concentrations were above 10% of nominal on all occasions. However, it was noted that percentage nominal results were similar on all formulation days for the range finding and main study experiments, the percentage nominal reading of all formulations was close to 120% of nominal indicating consistency of formulation. As no clinical signs were observed at the limit dose of 2000 mg/kg bw/day and the formulations analysis indicated achieved concentrations above 100%, these data were considered acceptable and the study data valid.

The results of the stability and homogeneity analysis indicated that the samples were homogenous and stable following storage at room temperature for a 24 hour period.

Mice treated with fluopicolide at 2000 mg/kg bw/day exhibited a group mean ratio of PCE to NCE which was similar to the value for the vehicle control group and which lay within the historical control data range (historical control data range: 0.38–1.67).
A higher number of micronucleated PCE as expected were noted in one control animal (animal no. 119: 2.25 micronucleated PCE/1000 cells). However, this was observed in just one animal in a total of six and all other vehicle control animals exhibited numbers of micronucleated PCE that fell within the expected distribution. The vehicle control data was therefore considered valid.

The group mean frequency of micronucleated PCE (MN PCE) in the test substance treated group was slightly increased compared to the concurrent negative control group value (1.50 ± 0.84 and 0.88 ± 0.70, respectively), but without any statistical significance. As such the protocol criteria for a positive result were not met. This is supported by the fact that a high number of MN PCE (9 MN PCE per 4000 analysed) was also detected in one control animal (see above) and that the group mean value of the positive control was significantly higher than the mean value of the fluopicolide treatment group (approx. 19 MN PCE compared to 1.5 MN PCE in the fluopicolide group).

An overview is given in Table 5.4.2- 4.

Table 5.4.2- 4: Individual and group mean PCE/NCE ratios and incidences of micronucleated PCE

Treatment group	Animal No.	PCE count	NCE count	Ratio PCE/NCE	No. MN PCE	No. MN PCE / 1000
Negative control (vehicle)	115	1089	991	1.10	3	0.75
	119	1132	959	1.18	9	2.25
	109	1045	1055	0.99	1	0.25
	101	1063	998	1.07	2	0.50
	102	980	1140	0.86	3	0.75
	112	898	1139	0.79	3	0.75
	Mean (± SD)	-	-	**1.00**	-	**0.88 ± 0.70**
Fluopicolide (2000 mg/kg bw/day)	106	1127	955	1.18	2	0.50
	116	1100	960	1.15	5	1.25
	103	986	1085	0.91	11	2.75
	108	1049	1012	1.04	3	0.75
	105	1049	1116	0.94	7	1.75
	104	1080	997	1.08	8	2.00
	Mean (± SD)	-	-	**1.05**	-	**1.50 ± 0.84**
Positive control (Cyclophosphamide, 40 mg/kg bw)	117	1116	1012	1.10	65	16.25
	107	1025	1037	0.99	84	21.00
	114	1087	1142	0.95	91	22.75
	118	1155	1001	1.05	71	17.75
	111	1005	1004	1.00	98	24.50
	113	1103	1064	1.04	46	11.50
	Mean (± SD)	-	-	**1.02**	-	**18.96± 4.77****
Historical negative control data (range)#	-	-	-	0.99 (0.38–1.67)	-	0.40

PCE: polychromatic erythrocytes
NCE: normochromatic erythrocytes
MN: micronucleated
**: $p \leq 0.01$
#: Historical control data based on results from 72 males from 11 studies

III. Conclusion

It is concluded that treatment of mice with fluopicolide at 2000 mg/kg bw/day (the recommended maximum dose level for *in vivo* cytogenetic studies according to current regulatory guidelines) resulted in a group mean frequency of micronucleated PCE in the test substance treated group that was slightly increased compared to the concurrent negative control group value, but was not statistically different. As such the protocol criteria for a positive result were not met which is supported by the fact that a high number of MN PCE was also detected in one control animal.

Assessment and conclusion by applicant:

The study was conducted in accordance with OECD TG 474 and is acceptable to assess the clastogenic potential of fluopicolide with the caveat of the deviations described (no bone marrow exposure). However, exposure was shown in an additional micronucleus test *in vivo* with i.p. administration ([redacted] 2003; M-223119-01-1) which showed no indication of a clastogenic effect of fluopicolide. Therefore, the results of the present study are acceptable as supplementary information.

Data Point:	KCA 5.4.2/03
Report Author:	[redacted]
Report Year:	2003
Report Title:	Micronucleus-test on the male mouse Code: AE C638206
Report No:	C037549
Document No:	M-223119-01-1
Guideline(s) followed in study:	Commission Directive: 2000/32/EC B.12. (2000); OECD 474 (1997); US-EPA 712-C-98-226, OPPTS 870.5395 (1998)
Deviations from current test guideline:	2000 rather than 4000 immature erythrocytes were analysed
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

The micronucleus test was employed to investigate fluopicolide in male NMRI mice for a possible clastogenic effect on the chromosomes of bone marrow erythroblasts.

The known clastogen and cytostatic agent cyclophosphamide served as positive control.

Male mice treated with fluopicolide received two intraperitoneal administrations of 150, 300 or 600 mg/kg bw, separated by 24 hours. Males of the positive control received a single intraperitoneal treatment with 20 mg/kg bw cyclophosphamide. Intraperitoneal administration as application route was chosen because bone marrow exposure could not be verified after oral administration (see [redacted] 2005; M-197261-02-1 and [redacted] 2003; M-219364-01-1).

The femoral marrow of all groups was prepared 24 hours after the last administration.

Males treated twice with fluopicolide in doses up to and including 600 mg/kg bw showed symptoms of toxicity after administration starting at 150 mg/kg. These symptoms demonstrate relevant systemic exposure of males to fluopicolide. However, all males survived until the end of the test.

There was an altered ratio between polychromatic and normochromatic erythrocytes. This finding demonstrates relevant systemic exposure of the animals to fluopicolide.

After two intraperitoneal treatments of males with doses up to and including 600 mg/kg bw no indications of a clastogenic effect of fluopicolide were found.

Cyclophosphamide, the positive control, had a clear clastogenic effect, as is shown by the biologically relevant increase in polychromatic erythrocytes with micronuclei. The ratio of polychromatic to normochromatic erythrocytes was not altered.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 (fluopicolide)
Purity:	99.4%
Batch no.:	OP2350005

2. Vehicle and/or positive control

Vehicle:	0.5% Cremophor
Positive control:	Cyclophosphamide

3. Test animals

Species:	Male mice
Strain:	Hsd/Win: NMRI
Age:	Approx. 6-12 weeks
Weight at start:	37-42 g
Source:	[redacted]
Acclimation period:	Yes
Diet:	Fixed formula feed 3883 (10 mm cubes), produced according to specification by Provimi Kliba SA, CH-4303 Kaiseraugst
Water:	Tap water in polycarbonate bottles, 300 ml volume
Housing:	Singly in type I cages, bedding of soft wood granules, type BK8/15 (J. Rettenmaier & Söhne, Fullstoff-Fabriken, 73494 Ellwangen-Holzmühle)
Temperature:	22±1.5 °C
Humidity:	40-70%
Air changes:	10/hour
Photoperiod:	12 hours

4. Test substance doses

Intraperitoneal administration as application route was chosen because bone marrow exposure could not be verified after oral administration (see [redacted]; 2005; M-197261-02-1 and [redacted] 2003; M-219364-01-1). The selection of the fluopicolide doses was based on a pilot test. This pilot test was performed in the laboratory which conducted the main study using animals of the same source, strain and age. Groups consisting each of three males and three females received two intraperitoneally injections separated by 24 hours. The following doses were used: 400 and 1000 mg/kg bw fluopicolide. In males the following symptoms were recorded for up to at least 48 hours after the second application, starting at 400 mg/kg bw: apathy, roughened fur, loss of weight, reduced body temperature, sternal recumbency, spasm, periodically stretching of body, difficulty in breathing and slitted eyes. In addition, 2 of 3 males died in the 1000 mg/kg bw group. In females the following symptoms were recorded for up to at least 48 hours after the second application, starting at 400 mg/kg bw: apathy, uncoordinated movement, roughened fur, loss of weight, reduced body temperature, sternal recumbency, spasm, twitching, periodically stretching of body, difficulty in breathing and slitted eyes. In addition, 2 of 3 females died in the 1000 mg/kg bw group.

Based on these findings, a dose of 600 mg/kg bw fluopicolide was chosen as MTD for males. Due to the results of the dose range finder it is concluded, that there are no substantial differences between sexes in toxicity. Therefore, no females were used.

B. Test performance

Experimental phase: September 3 to October 17, 2003

1. Treatment and sampling times

The study design of the main study was as follows.

Table 5.4.2- 5: Micronucleus test design

Experimental group	Dose in mg/kg bw	No. of animals	Route and number of applications	
Negative control	0	5	i.p.	2
Fluopicolide	150	5	i.p.	2
	300	5	i.p.	2
	600	5	i.p.	2
Positive control (Cyclophosphamide)	20	5	i.p.	1

Male mice (five animals/group) treated with fluopicolide (batch OP235005, purity 99.4%) received two intraperitoneal administrations of 150, 300 or 600 mg/kg bw separated by 24 hours. Males of the positive control received a single intraperitoneal treatment with 20 mg/kg bw cyclophosphamide.

2. Tissues and cells examined

At least one intact femur was prepared from each sacrificed animal (not pre-treated with a spindle inhibitor) (Schmid's method). A suitable instrument was used to sever the pelvic bones and lower leg.

The femur was separated from muscular tissue. The lower-leg stump, including the knee and all attached soft parts, was separated in the distal epiphyseal cartilage by a gentle pull at the distal end.

The proximal end of the femur was opened at its extreme end with a suitable instrument, e.g. fine scissors, making visible a small opening in the bone-marrow channel.

A suitable tube was filled with sufficient foetal calf serum. A small amount of serum was drawn from the tube into a suitable syringe with a thin cannula. The cannula was pushed into the open end of the marrow cavity. The femur was then completely immersed in the calf serum and pressed against the wall of the tube, to prevent its slipping off. The contents were then flushed several times and the bone marrow was passed into the serum as a fine suspension. Finally, the flushing might be repeated from the other end, after it had been opened. The tube containing the serum and bone marrow was centrifuged in a suitable centrifuge at approximately 1000 rpm for five minutes. The supernatant was removed with a suitable pipette (e.g. Pasteur pipette), leaving only a small remainder. The sediment was mixed to produce a homogeneous suspension.

One drop of the viscous suspension was placed on a well-cleaned slide and spread with a suitable object, to allow proper evaluation of the smear. The labelled slides were dried overnight. If fresh smears needed to be stained, they needed to be dried with heat for a short period.

The smears were stained automatically with an Ames Hema-Tek Slide Stainer from the Miles Company. The slides were then "destained" with methanol, rinsed with deionized water, and left to dry.

Following this treatment, the smears were transferred to a holder. A cuvette was filled with xylene, into which the holder was immersed for approximately ten minutes. The slides were removed singly (e.g. with tweezers) to be covered. A small amount of covering agent was taken from a bottle with a suitable object (e.g. glass rod) and applied to the coated side of the slide. A cover glass was then placed in position without trapping bubbles. The slides were not evaluated until the covering agent had dried.

3. Evaluation

Coded slides were evaluated using a light microscope at a magnification of about 1000. Micronuclei appear as stained chromatin particles in the anucleated erythrocytes. They can be distinguished from artifacts by varying the focus. Normally, 2000 polychromatic erythrocytes were counted per animal. The incidence of cells with micronuclei was established by scanning the slides in a meandering pattern.

It is expedient to establish the ratio of polychromatic to normochromatic erythrocytes for two reasons:

1. Individual animals with pathological bone marrow depressions may be identified and excluded from the evaluation.
2. An alteration of this ratio may show that the test compound actually reaches the target.

Therefore, the number of normochromatic erythrocytes per 2000 polychromatic ones was noted. If the ratio for a single animal amounts to distinctly more than 6000 normochromatic erythrocytes per 2000 polychromatic ones, or if such a ratio seems likely without other animals in the group showing similar effects, then the case may be regarded as pathological and unrelated to treatment, and the animal may be omitted from the evaluation. A relevant, treatment-related alteration of the ratio polychromatic to normochromatic erythrocytes can only be concluded if it is clearly lower for a majority of the animals in the treated group than in the negative control.

In addition to the number of normochromatic erythrocytes per 2000 polychromatic ones, the number of normochromatic erythrocytes showing micronuclei was also established. This information is useful in two ways. Firstly, it permits the detection of individuals already subject to damage before the start of the test. Secondly, combined with the number of micronucleated polychromatic erythrocytes, it permits a representation of the time-effect curve for positive substances.

An increase in the number of micronucleated normochromatic erythrocytes, without a preceding increase in micronucleated polychromatic erythrocytes, is irrelevant to the assessment of a clastogenic effect, since normochromatic erythrocytes originate from polychromatic ones. Before an effect can be observed in normochromatic erythrocytes, there must be a much greater increase in micronucleated polychromatic erythrocytes, due to the "dilution effect" of the "old" cells, i.e. normochromatic erythrocytes already present at the start of the test and this effect would have been observed previously.

4. Evaluation criteria

An assay was considered acceptable if the figures of negative and positive controls were within the expected range, in accordance with the laboratory's experience and/or the available literature data.

5. Criteria for a positive response

A test is considered positive if there was a relevant and significant increase in the number of polychromatic erythrocytes showing micronuclei in comparison to the negative control.

A test was considered negative if there was no relevant or significant increase in the rate of micronucleated polychromatic erythrocytes. A test was also considered negative if there was a significant increase in that rate which, according to the laboratory's experience was within the range of historical negative controls.

In addition, a test was considered equivocal if there was an increase of micronucleated polychromatic erythrocytes above the range of attached historical negative controls, provided the increase was not significant and the result of the negative control was not closely related to the data of the respective treatment group. In this case, normally a second test will be performed.

6. Statistical methods

The fluopicolide group(s) with the highest mean (provided this superseded the negative control mean) and the positive control were checked by Wilcoxon's nonparametric rank sum test with respect to the number of polychromatic erythrocytes having micronuclei and the number of normochromatic erythrocytes. A variation was considered statistically significant if its error probability was below 5 % and the treatment group figure was higher than that of the negative control.

The rate of normochromatic erythrocytes containing micronuclei was examined if the micronuclear rates for polychromatic erythrocytes was already relevantly increased. In this case, the group with the highest mean was compared with the negative control using the one-sided chi²-test. A variation was considered statistically significant if the error probability was below 5 % and the treatment group figure was higher than that of the negative control. In addition, standard deviations (1s ranges) were calculated for all the means.

II. Results and Discussion

A. Micronucleus assay

After two intraperitoneal administrations of 150, 300 or 600 mg/kg bw fluopicolide treated males showed the following compound-related symptoms until sacrifice: apathy, roughened fur, loss of weight, spasm, periodically stretching of body and difficulty in breathing. These symptoms demonstrate relevant systemic exposure of males to fluopicolide. There was no substance-induced mortality. No symptoms were recorded for the control groups. No animals died in these groups.

An overview of the genotoxicity evaluation is given in Table 5.4.2- 6.

Table 5.4.2- 6: Group mean PCE/NCE ratios and incidences of micronucleated PCE and NCE

Treatment group	Dose (mg/kg bw)	No. of animals	Total no. PCE scored	No. NCE/ 2000 PCE ± SD	No. micronucleated cells/2000 ± SD	
					NCE	PCE
Fluopicolide	600	5	10000	4948 ± 1782*	2.1 ± 1.8	5.0 ± 2.9
	300	5	10000	3523 ± 1189	0.9 ± 0.3	3.8 ± 1.3
	150	5	10000	2085 ± 1217	2.9 ± 3.1	1.8 ± 0.8
	0	5	10000	2356 ± 929	4.2 ± 2.4	2.8 ± 1.1
Positive control (Cyclophosphamide)	20	5	10000	1815 ± 614	3.4 ± 2.0	14.4 ± 5.2**

* $p \leq 0.05$; ** $p \leq 0.01$ (non-parametric Wilcoxon ranking test)

As can be seen in Table 5.4.2- 6, the ratio of polychromatic to normochromatic erythrocytes in males was altered by the treatment with fluopicolide, being 2000:2356 (1s = 929) in the negative control, 2000:2085 (1s = 1217) in the 150 mg/kg bw group, 2000:3523 (1s = 1189) in the 300 mg/kg bw group and 2000:4948 (1s = 1782) in the 600 mg/kg bw group. Relevant variations were thus noted for males. This finding demonstrates bone marrow exposure of the males to fluopicolide.

No biologically important or statistically significant variations existed for males between the negative control and the groups treated intraperitoneally with fluopicolide, with respect to the incidence of micronucleated polychromatic erythrocytes. The incidence of these micronucleated PCE was 2.8/2000 (1s = 1.1) in the negative control group, and 1.8/2000 (1s = 0.8), 3.8/2000 (1s = 1.3) and 5.0/2000 (1s = 2.9) in the fluopicolide groups at 150, 300 and 600 mg/kg bw, respectively.

Similarly, no biologically significant variation between the negative control and fluopicolide groups in the number of micronucleated normochromatic erythrocytes was observed, since normochromatic erythrocytes originated from polychromatic ones.

The positive control cyclophosphamide caused a clear, statistically significant increase in the number of polychromatic erythrocytes with micronuclei. The incidence of micronucleated PCE was 14.4/2000 (1s = 5.2), which represents biologically relevant increases in comparison to the negative control and thus confirms the sensitivity of this study.

III. Conclusion

The results with fluopicolide gave no relevant indications of clastogenic effects for male mice after two intraperitoneal treatments with doses of up to and including 600 mg/kg bw.

The ratio of polychromatic to normochromatic erythrocytes was altered by treatment and thus confirmed relevant systemic bone marrow exposure.

In conclusion, there was no indication of a clastogenic effect of intraperitoneally administered fluopicolide in the micronucleus test in male mice, i.e. in a somatic test system *in vivo*.

Assessment and conclusion by applicant:

This study was conducted according to OECD TG 474 and is valid and acceptable to determine the clastogenic potential of fluopicolide *in vivo*. Fluopicolide was not clastogenic under the conditions of this study.

Data Point:	KCA 5.4.2/04
Report Author:	[redacted]
Report Year:	2000
Report Title:	In vivo rat liver unscheduled DNA synthesis (DNA repair) test Code: AE C638206 00 1C99 0005
Report No:	C010494
Document No:	M-197230-02-1
Guideline(s) followed in study:	OECD 486 (1997); US-EPA OPPTS 870.5550; UK EMS testing guidelines
Deviations from current test guideline:	Deviations from the current OECD guideline (486, 1997): None.
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Data Point:	KCA 5.4.2/05
Report Author:	[redacted]
Report Year:	2000
Report Title:	In vivo rat liver unscheduled DNA synthesis (DNA repair) test. Determination of suspension concentrations for an in vivo rat liver repair (UDS) study
Report No:	M-199118-01-1
Document No:	M-199118-01-1
Guideline(s) followed in study:	OECD 486; US-EPA OPPTS 870.5550
Deviations from current test guideline:	Deviations from the current OECD guideline (486, 1997): None.
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

The potential of technical fluopicolide to induce DNA damage and repair as evidenced by unscheduled DNA synthesis in rat hepatocytes following a single oral dose was tested.

Two groups of four male Sprague Dawley rats were given a single oral dose of either 0, 600 or 2000 mg/kg bw fluopicolide in 1% w/v aqueous methyl cellulose. The higher dose level corresponded to the international regulatory limit dose for such tests. A concurrent negative control group was treated with the vehicle (1% w/v aqueous methylcellulose) and a positive control group was treated with dimethyl nitrosamine at 4 mg/kg bw (for the 2 hour expression) or 2-acetylaminofluorene at 50 mg/kg bw (for the 14 hour expression). Hepatocytes were isolated by enzymatic dissociation at 2 or 14 hours after exposure of the animals to the test substance. Four animals were assessed at each experimental point with the exception that only two animals from the positive control group were assessed at each expression time.

The isolated hepatocytes were allowed to attach to glass coverslips and were cultured *in vitro* with (methyl-^{3}H)thymidine at 10 µCi/mL for four hours to 'radiolabel' DNA undergoing repair replication. The hepatocytes were 'chased' for 24 hours with unlabelled thymidine then they were fixed and processed for autoradiography. DNA repair was assessed by comparing the labelling levels of hepatocyte nuclei from treated animals with control values and with the accompanying cytoplasmic labelling levels (usually a total of 150 cells per animal were examined).

Fluopicolide did not cause any significant increases in either the gross nuclear grain count or the net nuclear grain count (i.e. the gross nuclear grain count minus the cytoplasmic grain count) at any dose level at either sampling time.

Positive control group animals showed a large statistically significant increase in the net nuclear grain count, which was accompanied by a large increase in the gross nuclear grain count.

Thus, it is concluded that fluopicolide did not induce unscheduled DNA synthesis (DNA repair) in this *in vivo* rat liver test.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 (fluopicolide)
Purity:	97.7%
Batch no.:	PP/241067/1 & PP/241024/2

2. Vehicle and/or positive control

Vehicle:	1% w/v aqueous methylcellulose
Positive control:	Dimethylnitrosamine (4 mg/kg bw for the 2 hour expression) 2- acetylaminofluorene (50 mg/kg bw for the 14 hour expression)

3. Test animals

Species:	Male rat
Strain:	Outbred albino Hsd/Ola Sprague-Dawley rats
Age:	Approx. 6 weeks
Weight at start:	140-160 g
Source:	[redacted].
Acclimation period:	Yes
Diet:	Special Diet Services rat and mouse maintenance diet No. 1 (RM1(E) SQC expanded pellet)
Water:	Tap water
Housing:	Plastic disposable cages with a stainless steel grid top and maintained in a controlled environment
Temperature:	19-25 °C
Humidity:	30-70%
Air changes:	20 times/hour
Photoperiod:	12 hours

4. Test substance doses

Information received from a previous experiment indicated that 2000 mg/kg bw, the international regulatory limit dose for the rat liver DNA repair (UDS) test, was tolerated. Therefore, a preliminary toxicity test was not performed.

Doses of 0, 600 and 2000 mg/kg bw were chosen for use in the DNA repair test.

B. Test performance

Experimental phase: May 25 to August 15, 2000

1. Treatment and sampling times

Doses of 0, 600 and 2000 mg/kg bw were chosen for use in the DNA repair test. More than the required minimum of four animals (two for positive controls) were treated at each experimental point to allow for possible mortalities or technical difficulties encountered during perfusion. Two expression times were utilised in order to allow for variations in the rate of absorption, metabolism and accumulation of DNA damage.

The experimental design is shown below.

Table 5.4.2- 7: UDS *in vivo* test design

Experimental group	Dose in mg/kg bw	No. of animals	
		2 hour*	14 hour*
Negative control	0	4 (5)	4 (5)
Fluopicolide	600	4 (5)	4 (5)
	2000	4 (5)	4 (5)
Positive control			
Dimethylnitrosamine	4	2 (3)	Not done
2-Acetylamino-fluorene	50	Not done	2 (3)

* No. of animals for hepatocyte cultures (no. of treated animals)

Solutions of the test substance were freshly prepared on the day of use (using identical methods for each phase of the test) and were diluted to the required concentration in 1% w/v aqueous methylcellulose obtained from Colourcon, batch number MK02012N01.

Stability and homogeneity of the test substance in the vehicle at concentrations of 60 and 200 mg/mL were determined via an HPLC method using UV detection. Mean results for concentration were within the range 87.3 – 108.8% (acceptable range ± 20% of nominal), with homogeneity at both concentrations on day 1 being within the range of 80 – 120% of nominal and stability declining by less than 12 % over 14 hours storage at room temperature.

All animals in all groups were dosed orally by gastric intubation with the standard dose volume of 10 mL/kg bw. Animals in the negative control group were treated with the vehicle, 1% w/v aqueous methylcellulose. Animals in the positive control group were treated orally with dimethylnitrosamine at 4 mg/kg bw for the 2 hour expression or 2-acetylaminofluorene at 50 mg/kg bw for the 14 hour expression.

2. UDS test

Hepatocyte isolation and culture:

At the appropriate time after exposure (2 or 14 hours) each animal was killed by exposure to an increasing concentration of carbon dioxide. The liver was exposed and the hepatic portal vein was cannulated using a 18 gauge 1¼" Angiocath intravenous catheter placement unit (B-D 382821). The liver was perfused by this cannulation and via a bubble trap using a peristaltic pump set at a flow rate of 10 mL/min.

Perfusing media were held in a water bath at approx. 42 °C to give a temperature of approx. 37 °C at the outlet. The liver was initially perfused with EGTA solution for 5 minutes to deplete the liver of calcium ions and reduce cellular adhesion. Excess pressure on the liver was avoided by making a small puncture in the subhepatic vena cava just below the right renal vein. The liver was allowed to drain freely throughout the perfusion. It was then perfused with collagenase solution for 10 minutes. The liver was then excised and placed in a petri dish with a further aliquot of collagenase solution. Liver cells were combed into suspension using forceps and scissors then filtered through nylon bolting cloth (200 µm mesh). The hepatocytes were partially purified by differential centrifugation and finally resuspended in Williams' medium E, complete (WEC).

A viable cell count was performed after diluting an aliquot of the cells with an equal volume of trypan blue solution. Normally, mean viability values of about 85% are routinely obtained in this laboratory.

The viability of the cultures is not an absolute determinant of the validity of the experiment, subsequent attachment and washing stages tend to remove non-viable cells. Results are largely independent of the initial viability of the cultures. The viable cell yield was also calculated.

The isolated cells were suspended in WEC at a density of approximately 0.2 x 10^6 cells/mL. This cell suspension was dispensed in 2 mL aliquots into the 35 mm diameter wells of multi-well tissue culture plates, each well containing a sterile 22 mm diameter No. 1½ glass coverslip. Twelve replicate cultures were initiated per animal. The cultures were incubated at 37 °C in a humid atmosphere containing 5% carbon dioxide for 90 minutes to allow hepatocytes to attach to the coverslips. After this attachment period the supernatant medium was removed and the cells were gently rinsed with one wash of Williams' medium E, incomplete (WEI).

The medium was then replaced with WEI containing high specific activity (methyl-^{3}H)thymidine (Amersham International TRK 686, batch number 200; specific activity 79.0 Ci/mmol) at a final activity of 10 µCi/mL. The cultures were incubated in this medium for a period of 4 hours. After this labelling period, the supernatant medium was removed and replaced by WEI containing 250 µM cold (unlabelled) thymidine (TdR). The cultures were then incubated for a 'chase' period of 24 hours. This additional culture period helps to wash out excess radiolabel and improves cell morphology thus facilitating subsequent grain count analysis of autoradiographs.

Cell harvest:

After the 24 hour cold chase with thymidine, coverslips with attached cells were removed from the culture medium, given three 5 minute washes in Hanks' balanced salts solution then fixed in 2.5 % v/v acetic acid in ethanol (2 washes each of 5 minutes) and allowed to air dry. They were mounted on glass microscope slides, with the cell layer uppermost, using DPX mountant The mountant was allowed to harden at approx. 37 °C.

Autoradiography:

Autoradiographs were prepared from six cultures per animal; slides from the remaining six cultures per animal were held temporarily in reserve in case of any technical problems with the first set of autoradiographs. Any unused slides were discarded on completion of the study.

Ilford K2 emulsion was applied to the slides in the dark room working under a 25 watt Kodak Number 1 red safelight. The emulsion was melted then diluted with an equal volume of water containing 4% v/v glycerol. The melted emulsion was placed in a dipping chamber and held at approx. 43 °C. Each slide was in turn dipped into the emulsion, withdrawn and held vertically for a few seconds, then excess emulsion was wiped off the back of the slide which was placed on a chilled metal plate for a few minutes to allow title emulsion to gel. The slides were partially dried in a gentle stream of air for approximately one hour then they were sealed in a light-tight box containing desiccated silica gel and allowed to dry overnight at room temperature. The silica gel was renewed and the autoradiographs exposed for a further 13 days at approx. 4 °C.

After the total exposure period of 14 days the autoradiographs were allowed to warm to room temperature for several hours then developed:

- Kodak D-19 developer 5 minutes at 15 °C
- 0.5 % v/v acetic acid 1 minute at 15-20 °C
- Kodak T-max 5 minutes at 20 °C
- Running tap water 20 minutes at 15-20 °C
- Distilled water 5 minutes at 15-20 °C

The slides were stained in Mayers' Haemalum (BDH 35060 4T) for 1 minute, rinsed in distilled water, washed in running tap water and then allowed to air dry.

Examination of the slides:

The stained autoradiographs were examined under code using a Zeiss Photomicroscope II connected to a dedicated Sorcerer (Perceptive Instruments) image analysis system via a solid state video camera.

Initially, autoradiographs were examined for signs of test substance-induced toxicity (e.g. pyknosis, reduced levels of radiolabelling). Three slides per animal were examined using high-magnification, oil-immersion optics; the remaining autoradiographs prepared from each animal were held as reserves in case of any technical problems with the three slides initially examined.

The image analyser was used in the area count mode and the count obtained was automatically converted to an equivalent grain count using a constant conversion factor of 0.1655 grains per pixel. This method is believed to give the most accurate assessment of labelling levels because actual grain counting methods do not take into account variation in grain size or overlapping of grains at the high density seen in the hepatocyte UDS system. Usually 50 hepatocytes over several widely separated randomly chosen fields of view from each of three cultures per animal were analysed. Only results from hepatocytes not in S-phase with a normal morphology (i.e. not pyknotic or lysed) without staining artifacts or debris were recorded. For each cell the number of silver grains overlying the nucleus was estimated using the image analysis system, then the number of silver grains in an equivalent and most heavily-grained, adjacent area of cytoplasm was estimated. The cytoplasmic grain count was subtracted from the gross nuclear grain count to give the net nuclear grain count. Mean grain counts were calculated for each slide examined. For slides showing a strong response, i.e. where the mean net grain count was in excess of 10, only 25 cells were examined. The number of cells with a net gram count of greater than or equal to five was recorded in the raw data.

3. Evaluation

Both gross and net nuclear grain counts for treated animals were compared with vehicle control counts using classical one-way analysis of variance followed by a Student's t test with an appropriate transformation of values if indicated by excessive variance (Snedecor and Cochran[19]).

A positive response is normally indicated by a substantial dose-associated statistically significant increase in the net nuclear grain count which is accompanied by a substantial increase in the gross nuclear grain count over concurrent control values.

A negative response is indicated by a mean net nuclear grain count which is not significantly greater than the concurrent control.

An equivocal response is obtained when the results do not meet the criteria specified for a positive or negative response.

4. Statistical methods

No details given in the report, only a publication given under References: Snedecor and Cochran[19]

II. Results and Discussion

A. DNA repair test

No mortalities or clinical signs of toxicity were obtained after treatment of the animals with fluopicolide, the vehicle control or the positive controls.

The results of the DNA repair test using the 2 hour and 14 hour expression periods are presented in Table 5.4.2- 8 and Table 5.4.2- 9, respectively.

Table 5.4.2- 8: Mean nuclear and cytoplasmic grain counts at the 2 hour expression

Treatment group	Dose (mg/kg bw)	Mean gross nuclear grain count	Mean cytoplasmic grain count	Mean net nuclear grain count
Fluopicolide	2000	12.8	20.6	-7.8
	600	13.1	20.5	-7.4
	0	14.9	23.6	-8.7
Positive control (Dimethylnitrosamine)	4	45.7***	13.7	32.0***
Historical vehicle control mean	-	16.2	-	-1.7

*** $p < 0.001$, statistically significant (Student's t test)

Results for 710 individual vehicle control animals with three to five animals in each control group, used in unrelated past experiments.

[19] Snedecor, G.W. and Cochran, W.G. (1967) Statistical Methods, 6th ed., Iowa State University Press

Table 5.4.2- 9: Mean nuclear and cytoplasmic grain counts at the 14 hour expression

Treatment group	Dose (mg/kg bw)	Mean gross nuclear grain count	Mean cytoplasmic grain count	Mean net nuclear grain count
Fluopicolide	2000	16.7	27.1	-10.4
	600	13.4	23.4	-10.0
	0	16.2	24.8	-8.6
Positive control (2-Acetylamino-fluorene)	4	41.9***	13.4	28.6***
Historical vehicle control mean [#]	-	13.2		-1.7

*** $p \leq 0.001$ statistically significant (Student's t test)

[#] Results for 710 individual vehicle control animals with three to five animals in each control group, used in unrelated past experiments.

None of the slides from any of the animals showed any obvious signs of toxicity. For animals treated with the vehicle control or the test substance a total of 150 hepatocytes were scored per animal. Since a positive response was obvious for animals treated with dimethylnitrosamine and 2-acetylaminofluorene, it was only necessary to score 75 hepatocytes per animal for the positive control group.
Animals treated with the test substance did not show any significant increase in the gross or net nuclear grain count at any dose level at either the 2 or 14 hour expression time. Grain counts were similar to vehicle control values and were within the range of historical control values (Table 5.4.2- 8, Table 5.4.2- 9, Figure 5.4.2- 1).

Animals treated with dimethylnitrosamine or 2-acetylaminofluorene showed a significant increase in the net nuclear grain count which was accompanied by a substantial increase in the gross nuclear grain count.

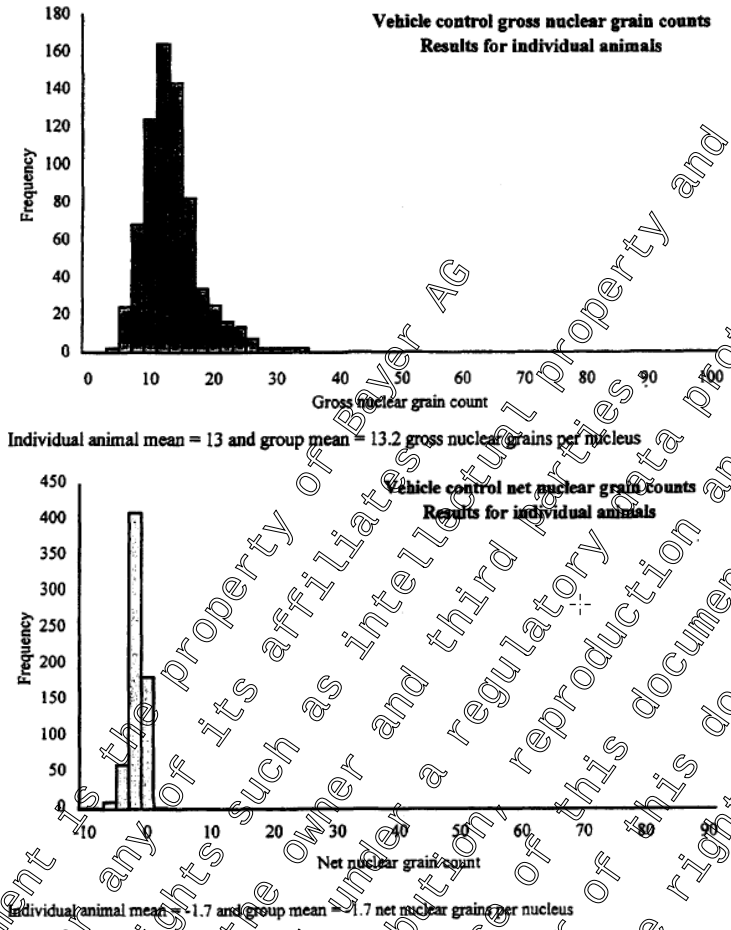


Figure 5.4.2- 1: Frequency distribution of historical vehicle control data (results for 710 individual vehicle control animals with three to five animals in each control group, used in unrelated past experiments).

III. Conclusion

Fluopicolide did not induce unscheduled DNA synthesis (DNA repair) in this *in vivo* rat liver test.

Assessment and conclusion by applicant:

The study was conducted according to OECD TG 406 and is valid and acceptable to assess the potential of fluopicolide to induce DNA damage and repair *in vivo*. Fluopicolide did not induce unscheduled DNA repair in the rat liver *in vivo* under the conditions of this study.

Data Point:	KCA 5.4.2/06
Report Author:	[redacted]
Report Year:	2018
Report Title:	In vivo mammalian alkaline comet assay - Fluopicolide, technical
Report No:	AE87WG.421MICH.BTL
Document No:	M-635020-01-1
Guideline(s) followed in study:	OECD 489 (2016); US-EPA 40 CFR 160 (FIFRA)
Deviations from current test guideline:	Deviations from the current OECD guideline (489, 2016): None.
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

The test substance fluopicolide was evaluated for its genotoxic potential in the Comet assay to induce DNA damage in liver and kidney cells of male mice. 1 % (w/v) methylcellulose (400 to 800 cPs) in deionized water was selected as the vehicle. Test and/or control substance formulations were administered at a dose volume of 10 mL/kg/dose by oral gavage. The dose levels tested were 500, 1000 and 2000 mg/kg bw.

The test substance gave a negative (non-DNA damaging) response in this assay in liver and kidney for males in % Tail DNA. None of the test substance-treated animal slides had significant increases in the % Tail DNA compared to the respective vehicle controls. The vehicle control % Tail DNA was within the testing facility's historical range, and the positive control showed a statistically significant increase in % Tail DNA compared to the vehicle control. Thus, all criteria for a valid assay were met for liver and kidney.

Under the conditions of this study, the administration of fluopicolide at doses up to and including 2000 mg/kg bw did not cause any significant increase in DNA damage in liver and kidney relative to the concurrent vehicle control.

Therefore, fluopicolide was concluded to be negative in the *in vivo* Comet Assay.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 (fluopicolide)
Purity:	98.2%
Batch no.:	AE C638206-01-29

2. Vehicle and/or positive control

Vehicle:	1% (w/v) methylcellulose (400 to 800 cPs) in deionized water
Positive control:	Methyl methanesulfonate (MMS)

3. Test animals

Species:	Mice (male)
Strain:	Hsd:ICR (CD-1) mice
Age:	Approx. 6 weeks
Weight at start:	23.2 – 36.5 g
Source:	[redacted]
Acclimation period:	Yes
Diet:	Certified laboratory rodent chow (Envigo 2018C Teklad Global 18% Protein Rodent Diet) ad libitum.
Water:	Tap water (U.S. EPA drinking water standards)
Housing:	Animals of the same sex were housed up to five per Micro-Barrier cage. Cages were placed on racks equipped with an automatic watering system and Micro-VENT full ventilation, HEPA filtered system
Temperature:	~22 °C (calculated from °F)
Humidity:	50 ± 20%
Air changes:	10 times/hour
Photoperiod:	12 hours

4. Test substance doses

Doses of 0, 500, 1000 and 2000 mg/kg bw were chosen for this *in vivo* study. Dose formulation analysis demonstrated that the prepared samples were 91.2, 86.9, and 82.0-82.9% of target, respectively, with ≤ 10.0% RSD; indicating that the formulations were accurately prepared and homogenous. No test item was detected in the vehicle control sample.

Methyl methanesulfonate (MMS) was administered at a dose level of 40 mg/kg/day.

B. Test performance

Experimental phase: May 30 to June 27, 2017.

1. Treatment times

All animals of the control and treatment groups were dosed on two consecutive days (Study Days 1 and 2) with the vehicle / with test substance. The second dose occurred approx. 21 hours after the first dose.

Animals of the positive control group were dosed with the positive control (Methyl methanesulfonate) once approx. 3 to 4 hours prior to euthanasia on day 2. Animals initially were treated (on Day 1 at T=0) with the test or control substances and euthanized at the appropriate time as described in Table 5.4.2- 10.

Table 5.4.2- 10: Comet assay design

Treatment group	Dose level (mg/kg bw/day)	Dose volume[A] (mL/kg/	Route of application[B]	No. of animals per sex	Euthanasia time (hours after treatment)
Negative control (vehicle)	0	10	Oral, gavage	6	3-4
Fluopicolide	500	10	Oral, gavage	6	3-4
	1000	10	Oral, gavage	6	3-4
	2000	10	Oral, gavage	6	3-4
Positive control (MMS)[C]	40	10	Oral, gavage	3	3-4

[A] Based upon individual body weight
[B] Using appropriately sized disposable polypropylene syringes with gastric intubation tubes (needles). The route has been routinely used and is widely-accepted for use in the mammalian alkaline comet assay
[C] Animals dosed only once on day 2.

2. Tissue collection for Comet assay:

All animals were euthanized 3 to 4 hours after the last dose (Study Day 2) by CO_2 asphyxiation, and then, the following was performed:

- Animals were dissected and the liver and one kidney were removed and collected.
- A section of the liver and kidney were cut and placed in formalin for possible histopathology analysis.
- Another section of the liver and remaining kidney was placed in chilled mincing solution (Hanks' balanced salt solution with EDTA and DMSO) and was used in preparation of cell suspensions and Comet slides.

3. Preparation of cell suspensions and Comet slides:

A portion of each dissected liver and kidney were placed in 3 mL of cold mincing buffer, then the liver and kidney were finely cut (minced) with a pair of fine scissors to release the cells. Each cell suspension was strained through a Cell Strainer into a pre-labelled 50 mL polypropylene conical tube and the resulting liver and kidney cell suspensions were placed on wet-ice. An aliquot of the suspensions were used to prepare the Comet slides.

Preparation of slides:

From each liver and kidney suspensions, an aliquot of 2.5 µL was mixed with 75 µL (0.5 %) of low melting agarose. The cell/agarose suspension was applied to microscope slides commercially available pre-treated multi-well slides. Commercially purchased multi-well slides were used and these slides have 3 individual circular areas, referred to as wells in the text below. The slides were kept at 2-8 °C for at least 15 minutes to allow the gel to solidify. Slides will be identified with a random code that reflects the study number, group, animal number, and organ/tissue. At least two Trevigen, Inc 3-well slides were prepared per animal per tissue. Three wells were used in scoring and the other wells were designated as a backup. Following solidification of agarose, the slides were placed in jars containing lysis solution.

Lysis:

Following solidification of agarose, the slides were submerged in a commercially available lysis solution supplemented with 10% DMSO on the day of use. The slides were kept in this solution at least overnight at 2-8 °C.

Unwinding:

After cell lysis, slides/wells were washed with neutralization buffer (0.4 M tris hydroxymethyl aminomethane in purified water, pH ~7.5) and placed in the electrophoresis chamber. The chamber reservoirs were slowly filled with alkaline buffer composed of 300 mM sodium hydroxide and 1 mM EDTA (disodium) in purified water. The pH was > 13. All slides remained in the buffer for ~20 minutes at 2-10 °C and protected from light, allowing DNA to unwind.

Electrophoresis:

Using the same buffer, electrophoresis was conducted for 30 minutes at 0.7 V/cm, at 2-10 °C and protected from light. The electrophoresis time was constant for all slides.

Neutralization:

After completion of electrophoresis, the slides were removed from the electrophoresis chamber and washed with neutralization buffer for at least 10 minutes. The slides (gels) were then dehydrated with 200-proof ethanol for at least 5 minutes, then air dried for at least 4 hours and stored at room temperature with desiccant.

Staining:

Slides were stained with a DNA stain (i.e., Sybr-gold™) prior to scoring. The stain solution was prepared by diluting 1 µL of Sybr-gold™ stain in 15 mL of 1xTBE (tris-boric acid EDTA buffer solution).

4. Evaluation of DNA damage:

Three slides/wells per organ/animal were used. Fifty randomly selected non-overlapping cells per slide/well were scored resulting in a total of 150 cells evaluated per animal for DNA damage using the fully validated automated scoring system Comet Assay IV from Perceptive Instruments Ltd. (UK).

The following endpoints of DNA damage were assessed and measured:

- Comet Tail Migration; defined as the distance from the perimeter of the Comet head to the last visible point in the tail.
- % Tail DNA; (also known as % tail intensity or % DNA in tail); defined as the percentage of DNA fragments present in the tail.
- Tail Moment (also known as Olive Tail moment); defined as the product of the amount of DNA in the tail and the tail length [(% Tail DNA x Tail Length) / 100)].

Each slide was also examined for indications of cytotoxicity. The rough estimate of the percentage of "clouds" was determined by scanning 150 cells per animal, when possible (percentage of "clouds" was calculated by adding the total number of clouds for all slides scored, dividing by the total number of cells scored and multiplying by 100). The "clouds", also known as "hedgehogs", are a morphological indication of highly damaged cells often associated with severe genotoxicity, necrosis or apoptosis. A "cloud" is produced when almost the entire cell DNA is in the tail of the comet and the head is reduced in size, almost non-existent. "Clouds" with visible gaps between the nuclei and the comet tail were excluded from comet image analysis.

The Comet slides, which are not permanent (the slides can be affected/damaged by environmental storage conditions), will be discarded prior to report finalization.

5. Histopathology Evaluation

A portion of each dissected tissue was placed in formalin (10% neutral-buffered formalin) for possible histopathology analysis. Per the study protocol, histopathology evaluation was not performed since biologically significant increases in DNA damage were not observed.

All unused tissues/slides/blocks and tissue samples saved for histopathology will be discarded prior to finalization of the report.

6. Statistical Analysis

The median value of 150 counts of % Tail DNA, Tail moment and Tail migration were determined and presented for each animal in each treatment group for each organ. The mean and standard deviation of the median values only for % Tail DNA were presented for each treatment group. Statistical analysis was performed only for % Tail DNA.

In order to quantify the test substance effects on DNA damage, the following statistical analysis was performed:

- The use of parametric or non-parametric statistical methods in evaluation of data was based on the variation between groups. The group variances for % Tail DNA generated for the vehicle and test substance groups were compared using Levene's test (significant level of $p \leq 0.05$). If the differences and variations between groups were found not to be significant, a parametric one-way ANOVA followed by a Dunnett's post-hoc test was performed (significant level of $p < 0.05$).
- A linear regression analysis was conducted to assess dose responsiveness in the test substance treated groups ($p \leq 0.01$).
- A pair-wise comparison (Student's T-test, $p \leq 0.05$) was used to compare the positive control group to the concurrent vehicle control group.

7. Criteria for Determination of a Valid Assay

The group mean for the % Tail DNA for each tissue analysed should ideally be within the distribution of the historical negative control database for that tissue, and the positive control group must be significantly greater than the concurrent vehicle control group ($p \leq 0.05$) and should be compatible with those observed in the historical positive control data base.

At least three test substance doses were tested for at least one sampling time. At least five animals per group were available for analysis (with the exception of the Comet positive control animal group).

At least 150 cells/organ/animal were scored for % Tail DNA. In addition, at least 150 cells per organ per animal were scored to determine the proportion of hedgehogs as an indication of cytotoxicity.

The maximum dose evaluated for Comets must:

a) be the MTD or MFD, or

b) in the absence of cytotoxicity or MFD, a dose of 2000 mg/kg bw/day (limit dose) was used.

8. Evaluation of Test Results

The test substance was considered to have induced a positive response if

a) at least one of the group mean for the % Tail DNA of the test substance doses exhibited a statistically significant increase when compared with the concurrent negative control ($p \leq 0.05$), and

b) when multiple doses were examined at a particular sampling time, the increase was dose-related ($p \leq 0.01$) and

c) results of the group mean or of the individual animals of at least one group were outside the distribution of the historical negative control database for that tissue.

The test substance was considered to have induced a clear negative response if none of the criteria for a positive response were met and there was direct or indirect evidence supportive of exposure of, or toxicity to, the target tissue.

If the response was neither clearly positive nor clearly negative, or in order to assist in establishing the biological relevance of a result, the data were evaluated by expert judgment and/or further investigations. Any additional work was only carried out following consultation with, and at the request of, the Sponsor.

In some cases, even after further investigations, the data set precluded making a conclusion of positive or negative, at which time the response was concluded to be equivocal. In such cases, the Study Director used sound scientific judgment and reported and described all considerations.

Biological significance of a positive, negative and equivocal result was based on the information on cytotoxicity at the target tissue. Where positive or equivocal findings were observed solely in the presence of clear evidence of cytotoxicity (e.g. histopathology evaluation, changes in clinical chemistry measures), the study was concluded as equivocal for genotoxicity unless there was enough information that was supportive of a definitive conclusion. In the case of a negative study outcome where there were signs of toxicity at all doses tested, further study at non-toxic doses may be advisable.

9. Electronic Data Collection Systems

Electronic systems used for the collection or analysis of data included but not limited to the following (version numbers are maintained in the system documentation):

Table 5.4.2- 11: Electronic Data Collection Systems

System	Purpose
- LIMS Labware System	- Test Substance Tracking
- Excel (Microsoft Corporation)	- Calculations/Randomization
- Minitab	- Statistics
- Kaye Lab Watch Monitoring System (Kaye GE)	- Environmental Monitoring
- BRIQS	- Deviations and audit reporting
- Comet Assay IV(Perceptive Instruments)	- Scoring slides

II. Results and Discussion

A. Comet assay

No mortalities occurred and no clinical sign of toxicity were obtained at doses of 500 and 1000 mg/kg bw. At 2000 mg/kg bw piloerection was observed. No effect on the body weight development was seen.

Liver:

The mean % Tail DNA in liver cells are summarized for each treatment group and presented in the following table.

Table 5.4.2- 12: % tail DNA (group mean) in liver cells following administrations of test substance

Samples collected 3 to 4 hours post-last dose			
Treatment (mg/kg bw)	**No. of animals**	**Group mean (% of Clouds)**	**Tail DNA (%)^ (Mean±SD)**
Negative control	6	0.3	0.68±0.42
Fluopicolide (500)	6	4.0	1.16±0.68
Fluopicolide (1000)	6	2.5	1.32±0.42
Fluopicolide (2000)	6	2.8	1.62±1.08
MMS (40)[B]	3	23.7	28.84±1.42*
HCD – negative control	2		0.12 – 3.51

[A] Mean of 3 or 6 animals
[B] Methyl methanesulfonate (MMS), positive control for Comet assay, orally administered only once at 3 to 4 hours prior to organ collection on day 2.
SD = Standard Deviation
HCD: Range of studies performed 2011 to 2015
* $p \leq 0.05$ (Student's t-test)

Median values for the % Tail DNA, Tail moment and Tail migration (µm) for liver cells are calculated per 150 cells for each animal and are presented in Table 5.4.2- 13.

Table 5.4.2- 13: DNA damage data in liver cells following administrations of test substance

Samples collected 3 to 4 hours post-last dose								
Treatment	Animal No.	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Negative control	51	0	0.13	24.67	0.52	0.12	19.87	0.49
			0.13	20.97	0.50			
			0.11	13.98	0.44			
	52	0	0.04	12.75	0.19	0.05	14.39	0.26
			0.08	16.86	0.39			
			0.03	13.57	0.21			
	53	0	0.14	26.32	0.55	0.16	27.00	0.70
			0.14	30.02	0.63			
			0.19	24.67	0.92			
	54	0	0.05	8.63	0.25	0.05	12.75	0.27
			0.04	12.75	0.23			
			0.06	16.86	0.32			
	55	0	0.31	17.27	1.68	0.22	18.09	1.24
			0.17	16.04	0.03			
			0.19	20.97	1.00			
	56	0	0.27	31.66	1.63	0.22	25.63	1.14
			0.18	24.26	0.72			
			0.24	20.97	1.06			
Fluopicolide (500 mg/kg bw)	57	3	0.18	31.25	0.92	0.18	25.90	0.93
			0.20	25.49	0.98			
			0.15	20.97	0.89			
	58	3	0.13	19.33	0.76	0.11	19.19	0.65
			0.08	19.33	0.45			
			0.14	18.91	0.73			
	59	3	0.20	23.85	0.85	0.21	22.20	1.02
			0.21	22.62	1.14			
			0.23	20.15	1.06			
	60	3	0.28	27.96	1.87	0.19	21.11	1.18
			0.16	16.86	0.74			
			0.14	18.50	0.92			
	61	3	0.38	25.08	1.39	0.46	26.45	2.49
			0.52	28.78	2.97			
			0.57	25.49	3.11			
	62	3	0.14	18.50	0.71	0.14	18.37	0.69
			0.09	17.68	0.44			
			0.20	18.91	0.92			

Samples collected 3 to 4 hours post-last dose								
Treatment	Animal No.	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Fluopicolide (1000 mg/kg bw)	63	2	0.32	18.50	2.17	0.38	17.13	2.58
			0.35	17.68	1.80			
			0.46	15.21	3.77			
	64	1	0.20	11.92	1.27	0.22	14.12	1.56
			0.19	13.98	1.30			
			0.28	16.45	1.50			
	65	2	0.08	14.39	0.55	0.10	16.31	0.57
			0.11	16.45	0.58			
			0.12	18.09	0.59			
	66	3	0.24	20.56	1.37	0.18	17.96	1.04
			0.16	18.09	0.95			
			0.12	15.21	0.79			
	67	3	0.12	17.68	0.91	0.24	19.33	1.68
			0.27	19.33	1.98			
			0.34	20.97	2.15			
	68	4	0.07	9.46	0.60	0.12	15.35	0.70
			0.13	16.04	0.78			
			0.17	20.56	0.72			
Fluopicolide (2000 mg/kg bw)	69	3	0.23	15.21	1.37	0.19	15.90	1.17
			0.19	14.39	1.25			
			0.15	18.09	0.90			
	70	3	0.01	11.10	0.05	0.03	11.38	0.20
			0.04	10.28	0.27			
			0.04	12.75	0.27			
	71	3	0.16	16.86	0.69	0.19	19.74	1.00
			0.19	23.44	1.08			
			0.22	18.91	1.22			
	72	4	0.44	22.20	2.20	0.42	19.74	2.53
			0.25	14.80	2.15			
			0.55	22.20	3.23			
	73	3	0.23	17.68	1.20	0.28	17.54	1.69
			0.20	16.45	1.46			
			0.42	18.50	2.40			
	74	1	0.44	23.44	2.37	0.55	22.07	3.16
			0.51	19.33	3.47			
			0.69	23.44	3.63			

Samples collected 3 to 4 hours post-last dose								
Treatment	Animal No.	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Positive control (MMS)[B]	75	15	6.03	42.35	30.21	6.33	43.31	29.77
			6.60	41.53	28.92			
			6.36	46.05	30.17			
	76	27	5.91	37.01	28.27	5.98	38.51	29.55
			5.70	38.65	29.31			
			6.33	39.88	31.09			
	77	29	4.00	39.47	21.64	5.42	43.72	27.20
			5.52	45.64	28.54			
			6.73	46.05	30.43			

A Mean of median of 150 cells scored per animal
B Orally administered only once at 3 to 4 hours prior to organ collection on day 2

The scoring results and a statistical analysis of data indicated the following:

The presence of 'clouds' in the test substance groups was ≤ 40%, which was higher than the % of clouds in the vehicle control group (0.3%).

Group variances for mean of medians of the % Tail DNA in the vehicle and test substance groups were compared using Levene's test. The test indicated that there was no significant difference in the group variance ($p > 0.05$); therefore, the parametric approach, ANOVA followed by Dunnett's post-hoc analysis, was used in the statistical analysis of data.

No statistically significant response in the % Tail DNA (DNA damage) was observed in the test substance groups relative to the concurrent vehicle control group (ANOVA followed by Dunnett's post-hoc analysis, $p > 0.05$).

No dose-dependent increase in the % Tail DNA was observed across three test substance doses (regression analysis, $p > 0.01$).

The positive control (Methyl methanesulfonate) induced a statistically significant increase in the mean % Tail DNA in liver cells as compared to the vehicle control groups (Student's t test, $p \leq 0.05$).

In the vehicle control groups, % Tail DNA was within the historical vehicle control range for the liver.

Kidney:

The mean % Tail DNA in kidney cells are summarized for each treatment group and presented in Table 5.4.2- 14.

Table 5.4.2- 14: % Tail DNA (group mean) in kidney cells following administrations of test substance

Samples collected 3 to 4 hours post-last dose			
Treatment (mg/kg bw)	**No. of animals**	**Group mean (% of Clouds)**	**Tail DNA (%)[A] (Mean±SD)**
Negative control	6	3.8	0.43±0.18
Fluopicolide (500)	6	1.5	0.29±0.08
Fluopicolide (1000)	6	1.3	0.27±0.17
Fluopicolide (2000)	6	2.3	0.24±0.11
MMS (40)[B]	3	75.0	19.62±4.20*
HCD – negative control	-	-	0.21 – 1.04

A Mean of 3 or 6 animals
B Methyl methanesulfonate (MMS), positive control for Comet assay, orally administered only once at 3 to 4 hours prior to organ collection on day 2.
SD:Standard deviation
HCD: Range of studies performed 2014 to 2015
* $p \leq 0.05$ (Student's t-test)

Median values for the % Tail DNA, Tail moment and Tail migration (µm) for kidney cells are calculated per 150 cells for each animal and are presented in Table 5.4.2- 15.

Table 5.4.2- 15: DNA damage data in kidney cells following administrations of test substance

Samples collected 3 to 4 hours post-last dose								
Treatment	**Individual Comet Assay Data**					**Mean Comet Assay Data[A]**		
	Animal No.	**Cloud %**	**Tail Moment**	**Tail Migration (µm)**	**Tail DNA (%)**	**Tail Moment**	**Tail Migration (µm)**	**Tail DNA (%)**
Negative control (vehicle)	51	6	0.06	10.11	0.27	0.09	13.63	0.43
			0.08	13.32	0.51			
			0.12	17.46	0.51			
	52	5	0.11	15.62	0.51	0.10	13.17	0.51
			0.06	10.57	0.35			
			0.13	13.32	0.67			
	53	1	0.02	11.03	0.10	0.04	11.33	0.18
			0.05	13.32	0.26			
			0.04	9.65	0.19			
	54	9	0.05	14.24	0.21	0.05	15.31	0.23
			0.06	16.54	0.25			
			0.04	15.16	0.22			
	55	1	0.08	17.92	0.35	0.12	19.91	0.62
			0.15	23.89	0.84			
			0.14	17.92	0.67			
	56	1	0.07	11.49	0.38	0.10	11.94	0.58
			0.06	11.03	0.26			
			0.18	13.32	1.10			

Samples collected 3 to 4 hours post-last dose								
Treatment	Animal No.	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Fluopicolide (500 mg/kg bw)	57	0	0.03	7.81	0.14	0.03	8.58	0.15
			0.01	11.03	0.06			
			0.04	6.89	0.24			
	58	2	0.07	15.16	0.33	0.06	13.32	0.32
			0.04	11.49	0.21			
			0.07	13.32	0.41			
	59	1	0.09	15.16	0.37	0.06	15.62	0.23
			0.05	16.54	0.23			
			0.04	15.16	0.20			
	60	2	0.04	15.16	0.20	0.05	12.10	0.26
			0.02	5.05	0.12			
			0.09	16.08	0.45			
	61	1	0.06	10.11	0.29	0.06	9.95	0.36
			0.06	7.35	0.44			
			0.06	12.40	0.35			
	62	2	0.06	6.89	0.28	0.08	12.10	0.35
			0.13	14.70	0.54			
			0.04	14.70	0.22			
Fluopicolide (1000 mg/kg bw)	63	0	0.06	14.70	0.33	0.11	14.70	0.57
			0.09	13.32	0.52			
			0.19	16.08	0.87			
	64	2	0.03	4.13	0.14	0.05	9.65	0.26
			0.04	9.65	0.18			
			0.09	15.16	0.47			
	65	0	0.02	6.43	0.13	0.05	8.27	0.29
			0.09	11.03	0.50			
			0.05	7.35	0.25			
	66	2	0.01	5.05	0.08	0.02	7.20	0.10
			0.02	6.89	0.12			
			0.02	9.65	0.10			
	67	2	0.03	9.65	0.23	0.05	11.64	0.25
			0.05	11.03	0.25			
			0.05	14.24	0.26			
	68	1	0.02	5.97	0.10	0.02	9.19	0.12
			0.03	12.40	0.13			

Samples collected 3 to 4 hours post-last dose								
Treatment	Animal No.	Cloud %	Individual Comet Assay Data			Mean Comet Assay Data[A]		
			Tail Moment	Tail Migration (µm)	Tail DNA (%)	Tail Moment	Tail Migration (µm)	Tail DNA (%)
Fluopicolide (2000 mg/kg bw)	69	5	0.02	9.65	0.13	0.03	11.64	0.15
			0.05	14.24	0.25			
			0.01	11.03	0.05			
	70	1	0.05	8.73	0.25	0.05	11.33	0.32
			0.07	12.40	0.49			
			0.04	12.86	0.21			
	71	0	0.01	7.81	0.05	0.01	6.43	0.07
			0.01	6.89	0.08			
			0.01	4.59	0.07			
	72	6	0.05	16.08	0.23	0.04	13.48	0.20
			0.04	11.94	0.19			
			0.04	12.40	0.17			
	73	1	0.10	15.62	0.43	0.08	16.31	0.36
			0.06	17.00	0.28			
	74	1	0.04	15.62	0.24	0.06	14.24	0.32
			0.04	12.86	0.18			
			0.09	14.24	0.54			
Positive control (MMS)[B]	75	11	2.77	33.54	17.17	2.94	35.38	16.93
			2.78	35.83	15.17			
			4.27	36.75	18.46			
	76	3	4.60	29.05	23.29	4.98	41.50	24.46
			5.26	40.43	25.75			
			5.07	45.02	24.34			
	77	1	2.84	32.62	15.74	3.14	34.46	17.46
			3.43	36.29	19.17			

A Mean of median of 150 cells scored per animal.
B Orally administered only once at 3 to 4 hours prior to organ collection on day 2.

The scoring results and a statistical analysis of data indicated the following:

The presence of 'clouds' in the test substance groups was ≤ 2.3%, which was lower than the % of clouds in the vehicle control group (3.8%).

Group variances for mean of medians of the % Tail DNA in the vehicle and test substance groups were compared using Levene's test. The test indicated that there was no significant difference in the group variance ($p > 0.05$); therefore, the parametric approach, ANOVA followed by Dunnett's post-hoc analysis, was used in the statistical analysis of data.

No statistically significant response in the % Tail DNA (DNA damage) was observed in the test substance groups relative to the concurrent vehicle control group (ANOVA followed by Dunnett's post-hoc analysis, $p > 0.05$).

No dose-dependent increase in the % Tail DNA was observed across three test substance doses (regression analysis, $p > 0.01$).

The positive control (Methyl methanesulfonate) induced a statistically significant increase in the mean % Tail DNA in kidney cells as compared to the vehicle control groups (Student's t test, $p \leq 0.05$).

In the vehicle control group, % Tail DNA was within the historical vehicle control range for the kidney.

These results indicate that all criteria for a valid test, as specified in the protocol, were met.

The differences in the incidences of "clouds" between vehicle control and fluopicolide treatment group in liver and kidney are considered incidental artefacts because there was no dose-response and no consistent trend (increased presence of clouds after fluopicolide exposure in liver, decreased presence of clouds after fluopicolide exposure in kidney).

III. Conclusion

Under the conditions of the assay described in this report, fluopicolide was concluded to be negative for the induction of DNA damage in liver and kidney.

Assessment and conclusion by applicant:

The study was conducted according to OECD TG 489 and is valid and acceptable to assess the genotoxic potential of fluopicolide *in vivo*. Fluopicolide did not induce DNA damage in the liver or kidney of male mice and was therefore not mutagenic under the conditions of this study.

CA 5.4.3 In vivo studies in germ cells

Since overall it was concluded that fluopicolide did not show a genotoxic potential, and since no evidence of an effect on germ cells was seen in other studies, an *in vivo* genotoxicity study in germ cells was not regarded as necessary.

CA 5.5 Long-term toxicity and carcinogenicity

The long term and/or carcinogenicity studies conducted with fluopicolide are summarized in Table 5.5-1. Fluopicolide was administered to Sprague Dawley **rats** in the diet at concentrations of 0, 50, 200, 750 or 2,500 ppm for **2 years**. After a 1-year treatment period, 20 animals/sex/group were killed for assessment of chronic toxicity. In addition, the recovery of any effects seen during the 52-week toxicity phase was assessed in a subsequent 13-week recovery period. After the 2-year treatment period, the carcinogenicity was evaluated from all oncogenicity phase animals (60 animals/sex/group). The main target organs for toxicity were the liver and the kidneys with increased liver and kidney weights at 750 and 2,500 ppm after 52 and/or 104 weeks of treatment in males and/or females. Histopathological examination after 52 weeks of treatment indicated a dose related increased incidence and severity (slight to moderate) of centrilobular hepatocyte hypertrophy and an increased incidence and/or severity of cortical tubular basophilia in the kidneys in males at 2500 ppm and to a lesser extend at 750 ppm. At 2,500 ppm, the change in the kidneys was associated with increased incidences of other degenerative changes, including hyaline droplets within the cortical tubules, hyaline tubular casts and granular medullary casts especially in the male animals. After completion of the 13-week recovery period, there was still a slight increase in the severity of cortical tubular basophilia in the kidneys of the males, with all other changes showing full recovery. After 104 weeks an increased incidence and/or severity of cystic degeneration and foci of alteration in males and an increased incidence of eosinophilic foci of alteration in females was additionally detected in the liver at 750 and 2,500 ppm. Secondary to the induced metabolic activity of the liver an increased incidence of cystic follicular cell hyperplasia in the thyroids of males was observed at these dose levels. Secondary to the increased metabolic activity of the liver an increased incidence of cystic follicular cell hyperplasia in the thyroids of males was recorded. In addition, degenerative changes with increased severities, proliferative changes were recorded in the kidneys after 104 weeks. Hyperplasia of the papillary epithelium at 2,500 and 750 ppm was present at an increased incidence and severity in females and this was usually associated with mineralisation of the papillary/pelvic epithelium (high dose level only). No treatment-related adverse changes were observed at dose levels of ≤ 200 ppm. Therefore, the No Observed Adverse Effect Level (NOAEL) for toxicity was 200 ppm in both males and females (equivalent to 8.4 and 10.8 mg/kg bw/day in males and females, respectively). Furthermore, there was no evidence of carcinogenicity with fluopicolide up to and including the dose level of 2,500 ppm (equivalent to 109.4 and 142.2 mg/kg bw/day, in males and females, respectively).

In a **mouse oncogenicity** study, fluopicolide was administered to C57/BL6 mice in the diet at concentrations of 0 (control), 50, 400 and 3,200 ppm for **78 weeks**. After 52-week treatment period, 10 animals/sex/group were killed for assessment of chronic toxicity. After 78-week treatment period, the carcinogenicity was evaluated from all oncogenicity phase animals (50 animals/sex/group). Fluopicolide administered daily for 78 weeks produced severe reduction of the body weight gain (-45% in males and -35% in females) at 3,200 ppm indicating that the Maximal Tolerated Dose (MTD) was reached. The target organ identified was the liver. Higher liver weights, enlarged liver, increased number of masses and nodules in the liver were observed at 400 and 3,200 ppm at 52 and 78 weeks. These changes were associated with hepatocellular hypertrophy at 52 and 78 weeks, and high incidence of altered cell foci at 3,200 ppm at 78 weeks. A high incidence of hepatocellular adenoma was observed at 3,200 ppm at 78 weeks in both males and females and to a lesser extend at 52 weeks in females. Therefore, the NOAELs are 50 ppm for toxicity (equivalent to 7.9 mg/kg bw/day and 11.5 mg/kg bw/day in males and females, respectively) and 400 ppm for carcinogenicity (equivalent to 64.5 mg/kg bw/day and 91.9 mg/kg bw/day in males and females, respectively). These benign liver tumours occurred only at the highest dose reaching the MTD (severe body weight gain reduction in high dose animals). Moreover, no tumours were observed in other mouse tissues and these tumours did not progress into malignant neoplasia during the lifespan of these animals, no increased incidence of hepatocellular carcinoma was observed in any groups after a 78-week treatment period. The mode of action for the increased incidence of liver adenomas was found to be CAR-mediated and thus, secondary to liver enzyme induction, like that of phenobarbital (see [redacted] 2006; M-275342-01-1 and chapter CA 5.8.2). This MOA is considered of no relevance in humans.

According to the CLP guidance (Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures Version 5.0 July 2017), a carcinogenicity classification of fluopicolide is **not warranted**.

Fluopicolide was discussed at the 53rd meeting of the Committee for Risk Assessment. It was agreed that classification for carcinogenicity was not warranted in accordance with the criteria laid out in Regulation (EC) No 1272/2008.

Table 5.5- 1: Summary of long term studies

Study	NOAEL	LOAEL	Findings at LOAEL	Reference
2 year combined toxicity and carcinogenicity study in rats 0, 50, 200, 750 or 2,500 ppm (equivalent to 0, 2.1, 8.4, 31.5, 109.4 / 0 2.8, 10.8, 41.0, 142.2 mg/kg bw/day (M/F))	200 ppm (8.4/10.8 mg/kg bw/day in M/F)	750 ppm (31.5/41.0 mg/kg bw/day in M/F)	↑ total protein concentration and ↓ A/G ratio (M/F) ↑ liver and kidney weights (M/F) ↓ incidence of mammary masses (F) ↑ increased incidence and/or severity of centrilobular hepatocyte hypertrophy (M/F), incidence and/or severity of cystic degeneration and foci of alteration (M) and ↑ increased incidence of eosinophilic foci of alteration (F) in the liver ↑ incidence of cortical tubular basophilia and hyperplasia of the papillary epithelium (M/F) ↑ incidence of cystic follicular cell hyperplasia in the thyroids (week 104) (M)	[redacted] 2003; M-225616-01-1 KCA 5.5/01 [redacted] 2005; M-263575-01-1 KCA 5.5/04
Carcinogenicity study in mice 0, 50, 400, or 3,200 ppm (equivalent to 0, 7.9, 64.5 / 551.0 / 0, 11.5, 91.9, 772.3 mg/kg bw/day M/F)	50 ppm (7.9/11.5 mg/kg bw/day in M/F)	400 ppm (64.5/91.9 mg/kg bw/day in M/F)	↑ liver weights (M/F) ↑ increased incidence of animals bearing liver masses and nodules (M) ↑ increased incidence of hepatocyte hypertrophy (M/F)	[redacted] 2003; M-225595-01-1 KCA 5.5/02 [redacted] 2005; M-263591-01-1 KCA 5.5/05
Fluopicolide - Assessment of hepatocellular proliferation and lack of carcinogenic potential	N/A	N/A	This position paper draws together the mechanistic studies and data available to demonstrate that the increased hepatic tumours in mice are of no relevance to humans.	[redacted] 2006; M-275342-01-1 KCA 5.5/03

M = male F = female

Data Point:	KCA 5.5/01
Report Author:	[redacted]
Report Year:	2003
Report Title:	Combined carcinogenicity and toxicity study by dietary administration to CD rats for 104 weeks AE C638206
Report No:	C038733
Document No:	M-225616-01-1
Guideline(s) followed in study:	OECD: 453 (1981); US-EPA OPPTS 870.4300 (1998); JMAFF Test 2-1-16; 12 Nohsan 8147; EEC Guideline B.33 (1988)
Deviations from current test guideline:	Method: none; Study: Deviations from the current OECD guideline (453, 2018): - Coagulating gland, Harderian gland, vagina and bone marrow were not sampled, fixed or examined histopathologically These minor deviation(s) are considered not to compromise the results and outcome of the study.
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Data Point:	KCA 5.5/04
Report Author:	[redacted]
Report Year:	2005
Report Title:	Historical control data for neoplasms in long-term studies in CD rats
Report No:	IRF.14.10.05
Document No:	M-263575-01-1
Guideline(s) followed in study:	OPPTS 870.4200
Deviations from current test guideline:	Deviations from the current OECD guideline (453, 2018): - Coagulating gland, Harderian gland, vagina and bone marrow were not sampled, fixed or examined histopathologically These minor deviation(s) are considered not to compromise the results and outcome of the study.
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

The carcinogenic and toxic potential of fluopicolide was assessed in a 104-week dietary study in CrlCD* (SD)IGS BR rats. Groups of 60 male and 60 female rats received in the diet at concentrations of 0, 50, 200, 750 or 2,500 ppm for 104 weeks. An additional 20 male and 20 female rats assigned to each group were sacrificed after completion of 52 weeks of treatment and comprised the Toxicity phase of the study. A further 10 male and 10 female rats assigned to each group were treated for 52-weeks, followed by a 13-week period without treatment to assess the reversibility of any treatment-related findings; these animals constituted the Recovery phase of the study.

The overall achieved dosages for the 52-week toxicity phase were 2.5, 9.8, 37.0 and 125.5 mg/kg bw/day for males and 3.3, 12.9, 48.7 and 163.6 mg/kg bw/day for females receiving 50, 200, 750 and 2,500 ppm, respectively. The overall achieved dosages for the 104-week carcinogenicity phase were 2.1, 8.4, 31.5 and 109.4 mg/kg bw/day for males and 2.8, 10.8, 41.0 and 142.2 mg/kg bw/day for females receiving 50, 200,750 and 2,500 ppm, respectively.

There was no effect of treatment upon mortality. At 2,500 ppm, there was a marked reduction in body weight gain and a reduction in food consumption during week one, in both sexes, with body weight gain continuing to be low in week 2. Subsequent body weight gain was generally lower than controls, particularly in females where food intake continued to be slightly low. At dose levels of 750 and 200 ppm, body weight gain was reduced in week 1 only, but there was no measured effect on food consumption.

There were no ophthalmoscopic changes that were attributable to treatment and no alteration in urinary composition. Low haemoglobin concentrations were recorded throughout the majority of the treatment period in males and females at 2,500 ppm. Occasionally associated with this was a reduction of haematocrit in males and in females and low erythrocyte counts in females. As a consequence of these changes, mean cell haemoglobin was consistently low in males and mean cell haemoglobin concentration and mean cell volumes were also low on most occasions in these animals. No toxicological significant changes in haematological parameters were observed at lower dose levels.

Treatment related biochemical changes in the blood plasma that were detected at 750 and 2,500 ppm comprised high total protein concentrations and low albumin to globulin ratios up to Week 52 in males and females and high albumin concentrations in Week 13 in males; high creatinine concentrations (2,500 ppm only) and a trend towards marginally high total cholesterol concentrations in males; high potassium and calcium concentrations in both sexes in Week 52 and 104. At 200 ppm, biochemical changes in the blood plasma were confined to high total protein concentrations in Week 13 and 26, associated in Week 26 with low albumin to globulin ratio, in males. None of the haematological or clinical chemistry changes were present at the end of the recovery period.

The main target organs for toxicity were the liver and the kidneys with increased liver and kidney weights at 750 and 2,500 ppm after 52 and/or 104 weeks of treatment in males and/or females. None of the changes apparent after 52 weeks of treatment were present after completion of the recovery period. After 104 weeks of treatment there was an increase of the thyroid weight at 2,500 ppm in males only. No relevant organ weight changes were detected at lower dose levels.

There were no treatment-related macroscopic changes after 52 weeks of treatment. In the carcinogenicity phase animals, when all animals were considered together, there was a lower incidence of mammary masses in females at ≥ 200 ppm.

Histopathological examination after 52 weeks of treatment indicated a dose related increased incidence and severity (slight to moderate) of centrilobular hepatocyte hypertrophy and an increased incidence and/or severity of cortical tubular basophilia in the kidneys in males at 2,500 ppm and to a lesser extent at 750 ppm. At 2,500 ppm, the change in the kidneys was associated with increased incidences of other degenerative changes, including hyaline droplets within the cortical tubules, hyaline tubular casts and granular medullary casts especially in the male animals. After completion of the 13 week recovery period, there was still a slight increase in the severity of cortical tubular basophilia in the kidneys of the males at 750 and 2,500 ppm, with all other changes showing full recovery. After 104 weeks in addition to centrilobular hepatocyte hypertrophy an increased incidence and/or severity of cystic degeneration and foci of alteration in males and an increased incidence of eosinophilic foci of alteration in females were detected in the liver at 750 and 2,500 ppm. Secondary to the induced metabolic activity of the liver an increased incidence of cystic follicular cell hyperplasia in the thyroids of males was observed at these dose levels. Furthermore, in addition to degenerative changes in the kidneys described after 52 weeks, proliferative changes were recorded in the kidneys after 104 weeks. Hyperplasia of the papillary epithelium at 2,500 and 750 ppm was present at an increased incidence and severity in females and this was usually associated with mineralisation of the papillary/pelvic epithelium (high dose level only). At the highest dose level there was an increased incidence and/or severity of acinar atrophic change in the pancreas in both sexes, evident in males as acinar atrophy and in females as acinar replacement by adipose tissue. An increased incidence of acinar atrophy, often associated with reduced colloid, was present in the prostate of males at 2,500 ppm only and is considered secondary to the decreased body weight at this dose level. There were no adverse treatment-related histopathological changes after 52 and 104 weeks of treatment at ≤ 200 ppm. The centrilobular hepatocytic hypertrophy in the livers of

2/60 males at 200 ppm observed during the histopathological examination of the carcinogenicity phase animals at 200 ppm is considered to be adaptive and not an adverse effect of treatment.

The administration of fluopicolide to CD rats at dietary concentrations of up to 2,500 ppm for 104 weeks did not provide any evidence of oncogenic potential.

It is concluded that the administration of fluopicolide to CD rats at dietary concentrations of up to 2,500 ppm for 104 weeks did not provide any evidence of oncogenic potential. The liver and kidneys were identified as target organs. A small number of findings were recorded for animals receiving 200 ppm. However, these were considered non-adverse. This dietary concentration is therefore assumed to be the No Observed Adverse Effect Level (NOAEL) of this study, equivalent to 8.4 mg/kg bw/day in males and 10.8 mg/kg bw/day in females. No treatment-related changes were detected in animals receiving 50 ppm and this dietary concentration is considered the No Observed Effect Level (NOEL) in this study, equivalent to 2.1 mg/kg bw/day in males and 2.8 mg/kg bw/day in females.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 (fluopicolide)
Purity:	95.9%
Batch no.:	OP2050046

2. Vehicle and/or positive control

Vehicle:	Diet

3. Test animals

Species:	Rat
Strain:	Crl:CD® (SD)IGS BR
Age:	40 to 44 days for males and 41 to 45 days for females
Weight at start:	159 to 234 g for males and 137 to 196 g for females
Source:	[redacted]
Acclimation period:	Yes
Diet:	Standard rodent diet (Rat and Mouse No. 1 Maintenance Diet from Special Diets Services Ltd., Witham, Essex, England), except when urine was being collected and overnight before routine blood sampling
Water:	Potable water taken from the public supply was freely available via polycarbonate bottles fitted with sipper tubes, except when urine was being collected
Housing:	The animals were housed four per sex per cage (Toxicity and Carcinogenicity phase animals) and three or four per sex per cage (Recovery phase animals), unless this number was reduced by mortality or isolation. The cages used were from RS Biotech, Finedon, Northamptonshire, England and were made of a stainless steel body with a stainless steel mesh lid and floor, and were suspended above absorbent paper, which was changed at appropriate intervals.

Temperature:	19 – 25 °C
Humidity:	40 to 70%
Air changes:	Each animal room was kept at positive pressure with respect to the outside by its own supply of filtered fresh air, which was passed to atmosphere and not re-circulated.
Photoperiod:	12 hours

B. Study design

1. In-life dates: August 23, 2000 to April 16, 2003

2. Animal assignment and treatment

The rats were randomized and assigned to the following test groups:

Table 5.5- 2: Study design

Group no.	Dose (ppm)	Number of males	Number of females
Toxicity phase			
1	0	20	20
2	50	20	20
3	200	20	20
4	750	20	20
5	2,500	20	20
Recovery phase (52 weeks + 13 weeks recovery)			
1	0	10	10
2	50	10	10
3	200	10	10
4	750	10	10
5	2,500	10	10
Carcinogenicity phase (104 weeks)			
1	0	60	60
2	50	60	60
3	200	60	60
4	750	60	60
5	2,500	60	60

The test substance fluopicolide was administered over a period of 52 weeks to animals in the Toxicity and Recovery phases and 104 consecutive weeks to animals in the Carcinogenicity phase.

Animals assigned to the Recovery phase completed a further thirteen weeks without treatment.

Treatment, and the recording of serial observations, continued for all surviving animals throughout the respective necropsy periods.

3. Diet preparation and analysis

Before treatment commenced, a reserve sample of the test substance was taken and placed in a well closed container and stored in the archives.

In order to demonstrate the integrity of the test substance under the storage conditions used at these laboratories, samples were returned to the Sponsor, for re-analysis, at the following intervals: October 2000, April 2001, September 2001, December 2001 and June 2002. The results of these analyses show that the quality of the test substance was satisfactory throughout the study.

Fluopicolide was prepared for administration as a series of graded concentrations in the diet. A pre-mix of a suitable dietary concentration was prepared by adding an approximately equal quantity of plain diet to the required weight of fluopicolide and mixing using a spoon. A further amount of plain diet that approximately equalled this mixture was then added and stirred into the mixture. This doubling-up procedure was followed until a visibly homogenous pre-mix of the required weight was achieved and the pre-mix blended in a Turbula Mixer. A second pre-mix was formulated from this first pre-mix using the doubling-up procedure described above and finally blended in a Turbula Mixer.

The 2,500 and 750 ppm formulations were prepared by direct dilution of the first pre-mix with further quantities of plain diet, and the 200 and 50 ppm formulations were prepared by direct dilution of the second pre-mix with plain diet. Blending was achieved by mixing in a Turbula Mixer. Blending in the Turbula Mixer was set at 100 cycles (approx. six minutes duration).

All dietary concentrations were expressed in terms of the test material as supplied.

Before treatment commenced, the suitability of the proposed mixing procedure was determined, and specimen formulations were analysed to assess the homogeneity and stability of the test substance in the diet. The homogeneity and stability were confirmed, with respect to the level of concentration, for fluopicolide in SDS Rat and Mouse No. 1 maintenance diet at 50 and 2,500 ppm.

Samples of each formulation prepared for administration in Weeks 1, 13, 26, 39, 52, 65, 79, 91 and 103 of treatment were analysed for achieved concentration of the test substance. The treated diets were mixed in batches for each group, different batches for each sex. Achieved concentration samples were taken alternately from batches designated for males and females commencing with males in Week 1. Samples were originally scheduled to be taken in Week 78 but were taken in Week 79. The method of analysis was an adaptation of a method supplied by the Sponsor.

All formulations were shown to be homogenous in the diet and stable at ambient temperature for up to 22 days. The mean concentrations of fluopicolide in Weeks 1, 13, 26, 39, 52, 65, 79, 91 and 103 were within 7% of intended and were therefore considered satisfactory.

4. Statistics

All statistical analyses were carried out separately for males and females. The analyses were carried out using the individual animal as the basic experimental unit.

The following data types were analysed at each time point separately:

Bodyweight, using gains over appropriate study periods
Blood chemistry, hematology and urinalysis
Organ weights, both absolute and adjusted for terminal bodyweight
Pathological findings, for the number of animals with and without each finding

For categorical data, including pathological findings, the proportion of animals was analysed using Fisher's Exact test for each treated group versus the control.

For continuous data, Bartlett's test was first applied to test the homogeneity of variance between the groups. Using tests dependent on the outcome of Bartlett's test, treated groups were then compared with the Control group, incorporating adjustment for multiple comparisons where necessary.

For body weight gains and organ weights, whenever Bartlett's test was found to be statistically significant, a Behrens-Fisher test was used to perform pairwise comparisons, otherwise a Dunnett's test was used.

The following sequence of statistical tests was used for clinical pathology data:

> If 75% of the data (across all groups) were the same value, for example c, then a frequency analysis was applied. Treatment groups were compared using a Mantel test for a trend in proportions and also pairwise Fisher's Exact tests for each dose group against the control both for i) values <c versus values >=c, and for ii) values <=c versus values >c, as applicable.
>
> If Bartlett's test for variance homogeneity was not significant at the 1% level, then parametric analysis was applied. If the F1 test for monotonicity of dose-response was not significant at the 1% level, Williams' test for a monotonic trend was applied. If the F1 test was significant, suggesting that the dose-response was not monotone, Dunnett's test was performed instead.
>
> If Bartlett's test was significant at the 1% level, then logarithmic and square-root transformations were tried. If Bartlett's test was still significant, then non-parametric tests were applied. If the H1 test for monotonicity of dose-response was not significant at the 1% level, Shirley's test for a monotonic trend was applied. If the H1 test was significant, suggesting that the dose-response was not monotone, Steel's test was performed instead.

Significant differences between control and treated groups were expressed at the 5% ($p<0.05$), 1% ($p<0.01$) or 0.1% ($p<0.001$) level. The following statistical cyphers were used throughout the report:

* - $p<0.05$; ** - $p<0.01$; *** - $p<0.001$ - using categorical or parametric tests

Details of the methods used are presented in the Statisticians Report.

C. Methods

1. Observations

Animals were inspected visually at least twice daily for evidence of ill-health or reaction to treatment.

Cages and cage-trays were inspected daily for evidence of ill-health amongst the occupants, such as loose faeces. Any deviation from normal was recorded at the time in respect of nature and severity, date and time of onset, duration and progress of the observed condition, as appropriate.

In addition, a more detailed weekly physical examination, which included palpation, was performed on each animal to monitor general health. Particular attention was paid to superficial or palpable swellings, for which the location, size, consistency, time of first observation and subsequent history were recorded.

During the acclimatisation and recovery periods, observations of the animals and their cages were made at least once per day.

Debilitated animals were observed carefully and, where necessary, isolated to prevent cannibalism.

Animals judged to be in extremis were killed. Animals were also killed to prevent unnecessary or prolonged suffering. A complete necropsy was performed in all cases.

2. Body weight

The weight of each rat was recorded on the day that treatment commenced (Week 0), each week for the first 16 weeks, then once every four weeks (to coincide with the end of a food consumption measurement period) and before necropsy. An exception to this was the body-weight recording for males that was scheduled originally for Week 68 but was re-scheduled to Week 67.

More frequent weight determinations were instituted, when appropriate, for animals displaying ill-health, so that the progress of the observed condition could be monitored.

3. Food intake

The weight of food supplied to each cage, that remaining and an estimate of any spilled was recorded each week for the first 16 weeks, then for one week in every four. An exception to this was the recording of food consumption for males that was scheduled originally for Week 68 but was re-scheduled to Week 67. From these records the mean weekly consumption per animal (g/rat/week) was calculated for each cage.

4. Ophthalmoscopic examination

Before treatment commenced, the eyes of all animals allocated to the study (including spare animals) were examined by means of a binocular indirect ophthalmoscope. Rejected animals were replaced with animals with no adverse ocular abnormality, selected from the spare animals for the study.

During Week 51 of treatment, the eyes of all surviving Toxicity phase animals from Groups 1 (control) and 5 (2,500 ppm) were examined. During Weeks 78 and 104, 20 male and 20 female animals from the Carcinogenicity phase (with the lowest surviving animal numbers) from Groups 1 and 5 were also examined.

Prior to each examination, the pupils of each animal were dilated using 0.5% tropicamide ophthalmic solution (Mydriacyl, Alcon Laboratories Ltd.). The adnexae, conjunctiva, cornea, sclera, anterior chamber, iris (pupil dilated), lens, vitreous and fundus were examined.

As no treatment-related changes were observed, the examination was not performed during the Recovery phase, nor was it extended to include animals of Groups 2, 3 and 4 (50, 200 and 750 ppm).

5. Laboratory investigations

<u>Haematology, peripheral blood</u>

During Weeks 13, 26 and 52, blood samples were obtained from all surviving Toxicity phase animals.

During Week 13 of recovery, blood samples were obtained from all surviving Recovery phase animals. During Weeks 78 and 104, blood samples were obtained from the 20 male and 20 female Carcinogenicity phase animals with the highest animal numbers remaining in each group. After overnight fasting, animals were held under light general anaesthesia induced by isoflurane and blood samples were withdrawn from the retro-orbital sinus. Blood samples (nominally 0.5 mL) were collected into EDTA as anticoagulant and examined.

The following parameters were measured using a Technicon H-1 haematology analyser:

Table 5.5- 3: Haematology

Haematocrit (Hct)	Differential leucocyte count
Haemoglobin concentration (Hb)	Neutrophils (N)
Erythrocyte count (RBC)	Lymphocytes (L)
Mean cell haemoglobin (MCH)	Eosinophils (E)
Mean cell haemoglobin concentration (MCHC)	Basophils (B)
	Monocytes (M)
Mean cell volume (MCV)	Large unstained cells (LUC)
Leucocyte count (WBC)	Platelet count (Plt)

Abnormal morphology was flagged by the Technicon analyser. The most common morphological changes, anisocytosis, micro/macrocytosis and hypo-hyperchromasia were recorded as slight, moderate or marked.

Blood film (prepared for all samples) - Romanowsky stain, examined for abnormalities by light microscopy, in the case of flags from the Technicon analyser. Confirmation of a written description from the blood film was made where appropriate.

Additional blood samples (nominally 0.5 mL) were taken into citrate anticoagulant and examined for prothrombin time (PT) - using an ACL 1000 Analyser and IL PT-Fibrinogen reagent, and Activated partial thromboplastin time (APTT) - using an ACL 1000 Analyser and IL APTT reagent.

Haematology, blood smears

During Weeks 52, 78 and 104 blood smears were prepared from samples obtained without the use of anaesthesia from the tail veins of all Carcinogenicity phase animals not subject to the peripheral haematology sampling described above. In Week 104, due to poor survival, smears were only obtained from a small number of females. Animals were not starved prior to sampling.

The smears prepared from animals in Groups 1 (control) and 5 (2,500 ppm) were examined as follows:

Differential leucocyte count - Romanowsky stain and direct visual count, differentiating among the following parameters:

Neutrophils
Lymphocytes
Eosinophils
Basophils
Monocytes
Abnormalities of the blood film

The smears obtained from the lower dose group animals were not examined as no blood disorder was suspected.

Blood chemistry

During Weeks 13, 26 and 52, blood samples were obtained from 10 male and 10 female Toxicity phase animals with the highest animal numbers remaining in each group.

During Week 13 of recovery, blood samples were obtained from all surviving Recovery phase animals. During Weeks 78 and 104, blood samples were obtained from 10 male and 10 female Carcinogenicity phase animals with the highest animal numbers remaining in each group.

Samples were taken, after overnight starvation, from the retro-orbital sinus with the animals held under light general anaesthesia induced by isoflurane. The blood samples (nominally 0.7 ml) were collected into lithium heparin as anticoagulant. All tubes were mechanically agitated for at least two minutes and the sample subsequently centrifuged at 3000 rpm for 10 minutes in order to separate the plasma. After separation, the plasma was examined using a Hitachi 917 Clinical Chemistry Analyser on the following parameters.

Table 5.5- 4: Blood chemistry

Alkaline phosphatase (ALP)	Sodium (Na)
Alanine aminotransferase (ALT)	Potassium (K)
Aspartate aminotransferase (AST)	Chloride (Cl)
Gamma-glutamyl transpeptidase (gGT)	Calcium (Ca)
Creatinine phosphokinase (CPK)	Inorganic phosphorus (Phos)
Total bilirubin (Bili)	Total protein (Total Prot)
Urea	Albumin (Alb)
Creatinine (Creat)	Albumin/globulin ratio (A/G Ratio) - calculated from total protein concentration and analyzed albumin concentration
Glucose (Glue)	
Total cholesterol (Chol)	
Trigtycerides (Trig)	

Urinalysis

During Weeks 12, 25 and 51, overnight urine samples were collected from 10 male and 10 female Toxicity phase animals with the highest animal numbers remaining in each group. During Week 13 of recovery, samples were collected from all surviving Recovery phase animals. During Weeks 77 and 103, samples were collected from 10 male and 10 female Carcinogenicity phase animals with the highest animal numbers remaining in each group.

Animals were placed in an individual metabolism cage without food or water at approx. 16.00 hours; urine was collected until approx. 08.30 hours the following day.

The individual samples were examined for the following characteristics:

Table 5.5- 5: Urinalysis

Appearance (App) - by visual assessment
Volume (Vol)
pH - using a Radiometer PHM 92 pH meter
Specific gravity (SG) - using Atago UR-1 digital refractometer
Protein (Prot) - using Hitachi 917 Clinical Chemistry Analyser
Glucose (Glue), ketones (Keto), bile pigments (Bill), blood pigments (Blood) by Multistix.

A microscopic examination of the urine sediment was performed. An aliquot of the urine sample was centrifuged and the resulting deposit spread on a microscope slide. The deposit was examined for the presence of the following:

Table 5.5- 6: Urine sediment analysis

Epithelial cells (Epi)
Leucocytes (Leuc)
Erythrocytes (RBC)
Crystals (Cryst)
Spermatozoa and precursors (Sperm)
Casts
Other abnormal components (Abn)

Urinalysis for investigation of metabolite(s)

During Week 58 of treatment urine samples were obtained from 10 male and 10 female Carcinogenicity phase animals with the lowest animal numbers remaining in Groups 1 and 5. Each animal was placed in an individual metabolism cage without food or water at approx. 16.00 hours; urine was collected until approx. 08.30 hours the following day. The urine was collected over dry ice and stored deep frozen (approx. -20 °C) until analysis.

6. Sacrifice and pathology

Animals killed during the study and those surviving until the end of their scheduled treatment or recovery period were killed by carbon dioxide asphyxiation. The sequence in which the animals were killed after completion of treatment or recovery was selected to allow satisfactory inter-group comparison.

Macroscopic pathology

All animals were subject to a detailed necropsy. After a review of the history of each animal, a full macroscopic examination of the tissues was performed. The cranial roof was removed to allow observation of the brain, pituitary gland and cranial nerves. After ventral mid-line incision, the neck and associated tissues and the thoracic, abdominal and pelvic cavities and their viscera were exposed and examined in situ. Any abnormal position, morphology or interaction was recorded.

The requisite organs were weighed and external and cut surfaces of the organs and tissues were examined as appropriate. Any abnormality in the appearance or size of any organ and tissue was recorded and the required tissue samples preserved in appropriate fixative.

Photographs of unusual findings were taken at the discretion of the necropsy supervisor. These photographs are not presented in this report but are retained in the archives. The retained tissues were checked before disposal of the carcass.

Organ weights

The following organs, taken from each animal killed after the scheduled treatment or recovery periods, were dissected free of adjacent fat and other contiguous tissue and the weights recorded:

Table 5.5- 7: Organ weights

Adrenals	Ovaries
Brain	Spleen
Epididymides	Testes
Heart	Thymus
Kidneys	Thyroid with parathyroids*
Liver	Uterus with cervix

* Weighed after partial fixation

Bilateral organs were weighed together. The weight of each organ was also expressed as a percentage of the body weight recorded immediately before necropsy.

Tissue sampling and fixation

Testes and epididymides were fixed in Bouin's fluid prior to transfer to 70% industrial methylated spirit and eyes were fixed in Davidson's fluid. The urinary bladder was initially inflated with Bouin's fluid. Samples (or the whole) of the other tissues listed below from all animals were preserved in 10% neutral buffered formalin.

The following organs/tissues were sampled.

Table 5.5- 8: Sampled organs and tissues

Adrenals	Oesophagus
Aorta - thoracic	Ovaries
Brain	Pancreas
Caecum	Pituitary
Colon	Pharynx #
Duodenum	Prostate
Epididymides	Rectum
Eyes	Salivary glands +
Femurs +	Sciatic nerves +
Head #	Seminal vesicles
Heart	Skeletal muscle – thighs -
Ileum	Skin
Jejunum	Spinal cord
Kidneys	Spleen
Lachrymal glands	Sternum
Larynx #	Stomach
Liver	Testes
Lungs	Thymus
Lymph nodes	Thyroid with parathyroids
- mandibular	Trachea
- mesenteric	Urinary bladder
- regional to masses	Uterus and cervix
Mammary area - caudal	
Nose #	

+ Only one processed for examination
# Not processed for examination

Samples of any abnormal tissues were also retained and processed for examination. In those cases where a lesion was not clearly delineated, contiguous tissue was fixed with the grossly affected region and sectioned as appropriate.

Samples of the head (including nasal cavity, paranasal sinuses and nasopharynx), the larynx, nose and pharynx and the remaining femur, salivary gland, sciatic nerve and skeletal muscle (thigh) were not examined histologically, but are retained against any future requirement for microscopic examination.

7. Histopathology

Tissue processing

Relevant tissues were subject to histological processing. Tissue samples were dehydrated, embedded in paraffin wax, sectioned at approximately four to five micron thickness and stained with haematoxylin and eosin, except the testes which were stained using a standard periodic acid/Schiff (PAS) method.

Those tissues subject to histological processing included the following regions:

Adrenals - cortex and medulla
Brain - cerebellum, cerebrum and midbrain
Femur with joint - longitudinal section including articular surface, epiphyseal plate and bone marrow
Heart - included auricular and ventricular regions
Kidneys - included cortex, medulla and papilla regions
Liver - section from all main lobes
Lungs - section from two major lobes, to include bronchi
Spinal cord - transverse and longitudinal section at the cervical, lumbar and thoracic levels
Sternum - included bone marrow
Stomach - included keratinised, glandular and antrum in sections
Thyroid - including parathyroids in section where possible
Uterus - uterus section separate from cervix section

For bilateral organs, sections of both organs were prepared. A single section was prepared from each of the remaining tissues required for microscopic pathology.

Microscopic examination

Microscopic examination was performed as follows:

- All tissues preserved for examination (as specified above) were examined for all animals killed or dying during the study.
- All tissues preserved for examination (as specified above) were examined for all animals of Groups 1 (control) and 5 (2,500 ppm) sacrificed on completion of the Toxicity and Carcinogenicity phases.
- The kidney, liver and lungs were examined for all animals of Groups 2, 3 and 4 (50, 200 or 750 ppm) sacrificed on completion of the Toxicity and Carcinogenicity phases and from all animals killed on completion of the Recovery phase.
- Tissues reported at macroscopic examination as being grossly abnormal were examined for all animals.

Findings were either reported as "present" or assigned a severity grade. In the latter case one of the following five grades was used - minimal, slight, moderate, marked or severe. A reviewing pathologist undertook a peer review of the microscopic findings.

II. Results and Discussion

A. Results

1. Clinical results

There was no effect of treatment upon mortality.

In the Toxicity/Recovery phases there was a total of seven males and 12 females killed or dying during the treatment period, and a total of one male and two females killed or dying during the recovery period.

Table 5.5- 9: Cumulative mortality in toxicity/recovery group animals at selected time points

Week	Dose level (ppm)									
	Males					Females				
	0	50	200	750	2,500	0	50	200	750	2,500
Toxicity phase										
13	0	0	2	0	0	0	1	1	0	0
26	0	0	2	0	0	2	2	2	2	0
27	0	0	3	0	0	4	2	2	2	0
38	0	0	4	0	0	4	2	2	2	0
51	0	0	4	0	0	4	2	3	2	0
52	0	0	5	0	0	4	2	3	2	1
Recovery phase										
41	0	0	0	0	1	0	0	0	0	0
45	1	0	0	0	1	0	0	0	0	0
R2	1	0	0	0	1	0	0	1	0	0
R8	1	0	0	0	1	0	0	1	0	1
R9	1	1	0	0	1	0	0	1	0	1

R: Recovery

In the carcinogenicity phase there was a total of 161 males and 192 females killed or dying during the treatment period. Statistical analysis of mortality among carcinogenicity phase animals indicated that there was no statistically significant difference between the control and treated groups ($p > 0.05$).

Table 5.5- 10: Cumulative mortality in carcinogenicity group animals at selected time points

Week	Dose level (ppm)									
	Males					Females				
	0	50	200	750	2,500	0	50	200	750	2,500
3	0	1	0	0	0	0	0	0	0	0
19	2	1	0	0	0	0	1	0	0	0
27	2	1	0	2	0	0	1	0	0	0
39	2	2	0	2	0	0	2	0	1	1
53	4	2	4	3	2	0	4	2	2	1
65	6	2	5	5	5	3	10	7	4	1
78	13	9	9	11	12	12	19	10	13	8
91	23	18	18	20	22	24	37	22	24	21
104	36	32	32	28	31	39	45	37	36	35

During the treatment periods (toxicity, recovery and carcinogenicity animals), there were incidences of perogenital staining brown staining on the dorsal body surface and brown staining of the pinnae in females at 750 and 2,500 ppm, but there was no a clear dose relationship. These signs tended to resolve during the recovery period, though in each case, only a low number of recovery phase animals had these signs on completion of the 52-week treatment period. This observation of a generalized reduction in grooming is not indicative of significant toxicity and is not considered adverse. There were no treatment-related signs amongst animals receiving 50 or 200 ppm.

In the carcinogenicity phase a total of 137 males and 208 females bore one or more palpable swellings during the treatment period. In females the number of animals with swellings and the total number of swellings were lower in treated groups than in the controls in a broadly dosage-related manner; this was particularly marked in females receiving 750 or 2,500 ppm, 77 and 79 swellings respectively, compared with 111 in the controls. However, this is not considered an adverse effect.

Table 5.5- 11: Group distribution, multiplicity and mean time of onset of palpable swellings in the carcinogenicity phase animals

Dose level (ppm)	Multiplicity#					No. of animals with swellings	Total no. of swellings	Mean time of onset (weeks)
	0	1	2	3	4			
Males								
0	36	13	10	1	0	24	36	73
50	36	14	8	0	2	24	38	73
200	31	12	9	6	2	29	56	72
750	32	15	5	5	3	28	55	69
2,500	28	18	10	3	1	32	52	71
Females								
0	9	21	14	7	9	51	111	74
50	15	18	15	6	6	45	102	72
200	19	18	11	4	8	41	90	78
750	25	14	11	7	3	35	77	79
2,500	24	15	13	5	5	36	79	79

Expressed as number of animals bearing the indicated number of swellings

2. Body weights

Absolute body weight was consistently lower compared to control at the highest tested dose in both sexes throughout treatment (week 104: -9% and -12% for males and females, respectively; Table 5.5-12).

Table 5.5- 12 provides information regarding absolute body weight measurements during the study.

Table 5.5- 12: Mean body weight at selected time points (g) *(% difference to control)*

	Dose level (ppm)							
	0	**50**		**200**		**750**	**2,500**	
Males								
Toxicity phase animals								
Wk 0	196	195	*(-0.5)*	196	*(±0.0)*	199	*(+1.5)*	197	*(+0.5)*
Wk 13	536	538	*(+0.4)*	534	*(-0.4)*	531	*(-0.9)*	503	*(-6.2)*
Wk 28	652	657	*(+0.8)*	658	*(+0.9)*	653	*(+0.2)*	625	*(-4.1)*
Wk 36	695	697	*(+0.3)*	698	*(+0.4)*	694	*(-0.1)*	664	*(-4.5)*
Wk 52	751	754	*(+0.4)*	756	*(+0.7)*	752	*(+0.1)*	723	*(-3.7)*
Recovery phase animals									
Wk R0	765	785	*(+2.4)*	773	*(+1.0)*	791	*(+3.4)*	733	*(-4.2)*
Wk R4	781	801	*(+2.6)*	788	*(+0.9)*	807	*(+3.3)*	743	*(-4.9)*
Wk R8	796	823	*(+3.4)*	800	*(+0.5)*	829	*(+4.1)*	767	*(-3.6)*
Wk R12	800	831	*(+3.9)*	813	*(+1.6)*	841	*(+5.1)*	778	*(-2.8)*
Carcinogenicity Phase									
Wk 0	198	197	*(-0.5)*	198	*(±0.0)*	197	*(-0.5)*	194	*(-2.0)*
Wk 13	553	559	*(+1.1)*	566	*(+2.4)*	548	*(-0.9)*	509	*(-8.0)*
Wk 28	667	671	*(+0.6)*	681	*(+2.1)*	651	*(-2.4)*	624	*(-6.4)*
Wk 36	712	721	*(+1.3)*	731	*(+2.7)*	696	*(-2.2)*	669	*(-6.0)*
Wk 52	778	788	*(+1.3)*	787	*(+1.2)*	754	*(-3.1)*	729	*(-6.3)*
Wk 64	820	826	*(+0.7)*	824	*(+0.5)*	799	*(-2.6)*	758	*(-7.6)*
Wk 76	834	857	*(+2.8)*	847	*(+1.6)*	829	*(-0.6)*	772	*(-7.4)*
Wk 96	832	862	*(+3.6)*	866	*(+4.1)*	839	*(+0.8)*	787	*(-5.4)*
Wk 104	817	825	*(+1.0)*	861	*(+5.4)*	795	*(-2.7)*	746	*(-8.7)*
Females									
Toxicity phase animals									
Wk 0	169	164	*(-3.0)*	165	*(-2.4)*	164	*(-3.0)*	165	*(-2.4)*
Wk 13	314	313	*(-0.3)*	308	*(-1.9)*	297	*(-5.4)*	281	*(-11.1)*
Wk 28	375	360	*(-4.1)*	357	*(-4.8)*	348	*(-7.2)*	324	*(-13.6)*
Wk 36	406	383	*(-5.7)*	381	*(-6.2)*	372	*(-8.4)*	343	*(-15.5)*
Wk 52	465	438	*(-5.8)*	438	*(-5.8)*	421	*(-9.5)*	387	*(-16.8)*
Recovery phase animals									
Wk R0	462	455	*(-1.5)*	438	*(-5.2)*	432	*(-6.5)*	389	*(-15.8)*
Wk R4	481	467	*(-2.9)*	460	*(-4.4)*	450	*(-6.4)*	418	*(-13.1)*
Wk R8	497	478	*(-3.8)*	472	*(-5.0)*	463	*(-6.8)*	437	*(-12.1)*
Wk R12	491	477	*(-2.9)*	475	*(-3.3)*	460	*(-6.3)*	442	*(-10.0)*

	Dose level (ppm)							
	0	50		200		750		2,500	
Carcinogenicity Phase								
Wk 0	165	166	*(+0.6)*	163	*(-1.2)*	164	*(-0.6)*	167	*(+1.2)*
Wk 13	318	312	*(-1.9)*	309	*(-2.8)*	310	*(-2.5)*	296	*(-6.9)*
Wk 28	373	358	*(-4.0)*	360	*(-3.5)*	362	*(-2.9)*	338	*(-9.4)*
Wk 36	398	384	*(-3.5)*	387	*(-2.8)*	390	*(-2.0)*	362	*(-9.0)*
Wk 52	461	443	*(-3.9)*	440	*(-4.6)*	451	*(-2.2)*	411	*(-10.8)*
Wk 64	497	488	*(-1.8)*	478	*(-3.8)*	493	*(-0.8)*	446	*(-10.3)*
Wk 76	538	502	*(-6.7)*	517	*(-3.9)*	527	*(-1.7)*	481	*(-10.6)*
Wk 96	556	513	*(-7.7)*	540	*(-2.9)*	577	*(+3.8)*	505	*(-9.2)*
Wk 104	554	508	*(-8.3)*	542	*(-2.2)*	558	*(+0.7)*	486	*(-12.3)*

Wk: Week; R: Recovery
According to study report, statistical analyses were not performed for absolute body weights (only for body weight gains, see following table)

When compared with the controls there was a statistically significant reduction in body weight gain in the first week of treatment in animals of both sexes receiving 2,500 and 750 ppm and in females receiving 200 ppm. When compared with the control gains, the Week 1 gains in animals receiving 2,500 ppm were reduced markedly in both sexes and this persisted to Week 2 in the Carcinogenicity phase animals. Subsequent weight gain by animals receiving 2,500 ppm tended to be lower than that of the controls, though the difference was less than was seen during the first two weeks of treatment. For males and females given 2,500 ppm, the overall weight gain was 5 and 25% lower than controls, respectively, at the end of the Toxicity phase (week 52) and 11 and 17% lower than controls at the end of the Carcinogenicity phase (week 104). At 200 and 750 ppm, the subsequent body weight gain of both sexes was similar to that of the controls.

In the recovery group, following the cessation of treatment at Week 52, females previously given 2,500 ppm and all male treatment groups gained more weight than the controls. However, a dose-relation was only seen for high-dose females.

The body weight gain of animals receiving 50 ppm and of males receiving 200 ppm were considered unaffected by treatment. The variations of weight gains in Week 1 for males receiving 200 ppm were small and inconsistent between the two phases of the study and were, therefore, not considered toxicologically significant.

Table 5.5- 13 gives an overview about the body weight gains.

Table 5.5- 13: Mean body weight gain (g) *(% difference to control)*

	Dose level (ppm)							
	0	**50**		**200**		**750**		**2,500**	
Males									
Toxicity phase animals									
Gain Week 0-1	57	56	*(-1.8)*	53	*(-7.0)*	50**	*(-12.3)*	35**	*(-38.6)*
Gain Week 1-2	50	53	*(+6.0)*	51	*(+2.0)*	51	*(+2.0)*	49	*(-2.0)*
Gain Week 0-52	556	559	*(+0.5)*	559	*(+0.5)*	554	*(-0.4)*	527	*(-5.2)*
Recovery phase animals									
Gain Week R0-R12	35	44	*(+25.7)*	40	*(+14.3)*	50*	*(+42.9)*	44	*(+25.7)*
Carcinogenicity phase animals									
Gain Week 0-1	52	53	*(+1.9)*	56*	*(+7.7)*	49	*(-6.1)*	35**	*(-32.7)*
Gain Week 1-2	54	52	*(-3.7)*	51	*(-5.6)*	51	*(-5.6)*	48**	*(-11.1)*
Gain Week 0-80	622	661	*(+6.3)*	657	*(+5.6)*	637	*(+2.4)*	573	*(-7.9)*
Gain Week 0-104	623	629	*(+1.0)*	665	*(+6.7)*	602	*(-3.4)*	555	*(-10.9)*
Females									
Toxicity phase animals									
Gain Week 0-1	28	32*	*(+14.3)*	18**	*(-35.7)*	17**	*(-39.3)*	13**	*(-53.6)*
Gain Week 1-2	18	17	*(-5.6)*	26**	*(+44.4)*	24**	*(+33.3)*	20	*(+11.1)*
Gain Week 0-52	297	273	*(-8.1)*	273	*(-8.1)*	257	*(-13.5)*	222**	*(-25.3)*
Recovery phase animals									
Gain Week R0-R12	29	22	*(-24.1)*	31	*(+6.9)*	28	*(-3.4)*	49	*(+69.0)*
Carcinogenicity phase animals									
Gain Week 0-1	25	23	*(-8.0)*	20**	*(-20.0)*	7**	*(-72.0)*	18**	*(-28.0)*
Gain Week 1-2	26	22*	*(-15.4)*	27	*(+3.8)*	25	*(-3.8)*	15**	*(-42.3)*
Gain Week 0-80	376	337	*(-10.4)*	353	*(-6.1)*	369	*(-1.9)*	316**	*(-16.0)*
Gain Week 0-104	390	346	*(-11.3)*	378	*(-3.1)*	396	*(+1.5)*	322	*(-17.4)*

R: Recovery
* $p \leq 0.05$; ** $p \leq 0.01$, statistically different to control

3. Food intake

Males and females receiving 2,500 ppm consumed less food than the controls in the first week of treatment (-13% and -7% for the toxicity phase males and females, respectively, and -20% and -7% for the carcinogenicity phase males and females, respectively). Thereafter, females receiving 2,500 ppm, particularly those in the toxicity phase, tended to consume slightly less food than the control animals in the majority of the weeks and, consequently, the total food intake of these animals was slightly low (-8% for the toxicity phase females and -5% for the carcinogenicity phase females).

In the recovery phase of the study all groups of previously treated animals consumed similar amounts of food as the control animals with the exception of females which had received 2500 ppm where the food intake remained slightly low.

Animals receiving 750 or 2,500 ppm scattered more food than the controls in the first week of treatment and in the carcinogenicity phase females this continued into Week 2 and 3 when it was also apparent in females receiving 200 ppm. From Week 4 of treatment the food scatter values for treated animals were similar to those of the controls.

Table 5.5- 14 gives an overview of the food consumption.

Table 5.5- 14: **Mean food consumption in g/animal** *(% difference to control)*

	Dose level (ppm)							
	0	**50**		**200**		**750**	**2,500**	
Males								
Toxicity phase animals								
Wk 1	199	200	*(+0.5)*	195	*(-2.0)*	192	*(-4.0)*	174	*(-12.6)*
Wk 13	179	177	*(-1.1)*	175	*(-2.2)*	177	*(-1.1)*	172	*(-3.9)*
Wk 28	198	199	*(+0.5)*	196	*(-1.0)*	198	*(±0.0)*	195	*(-1.5)*
Wk 36	192	196	*(+2.1)*	192	*(±0.0)*	194	*(+1.0)*	190	*(-1.0)*
Wk 52	180	194	*(+7.8)*	186	*(+3.3)*	183	*(+1.7)*	178	*(-1.1)*
Wk 1-52	10123	10227	*(+1.0)*	10170	*(+0.5)*	10162	*(+0.4)*	9890	*(-2.3)*
Recovery phase animals									
Wk 4	192	200	*(+4.2)*	188	*(-2.1)*	202	*(+5.2)*	198	*(+3.1)*
Wk 8	195	203	*(+4.1)*	193	*(-1.0)*	208	*(+6.7)*	213	*(+9.2)*
Wk 12	188	202	*(+7.4)*	188	*(±0.0)*	201	*(+6.9)*	196	*(+4.3)*
Wk 1-12	2300	2420	*(+5.2)*	2276	*(-1.0)*	2444	*(+6.3)*	2428	*(+5.6)*
Carcinogenicity phase animals									
Wk 1	205	207	*(+1.0)*	208	*(+1.5)*	197	*(-3.9)*	165	*(-19.5)*
Wk 13	197	196	*(-0.5)*	195	*(-1.0)*	190	*(-3.6)*	191	*(-3.0)*
Wk 28	198	191	*(-3.5)*	194	*(-2.0)*	190	*(-4.0)*	189	*(-4.5)*
Wk 36	194	196	*(+1.0)*	199	*(+2.6)*	185	*(-4.6)*	185	*(-4.6)*
Wk 52	196	196	*(±0.0)*	197	*(+0.5)*	183	*(-6.6)*	183	*(-6.6)*
Wk 64	191	199	*(+4.2)*	199	*(+4.2)*	192	*(+0.5)*	184	*(-3.7)*
Wk 76	200	200	*(±0.0)*	199	*(-0.5)*	202	*(+1.0)*	196	*(-2.0)*
Wk 96	204	209	*(+2.5)*	197	*(-3.4)*	213	*(+4.4)*	209	*(+2.5)*
Wk 104	197	192	*(-2.5)*	203	*(+3.0)*	192	*(-2.5)*	197	*(±0.0)*
Wk 1-104	20679	21101	*(+1.6)*	20975	*(+1.4)*	20487	*(-0.9)*	20255	*(-2.1)*
Females									
Toxicity phase animals									
Wk 1	144	146	*(+1.4)*	146	*(+1.4)*	147	*(+2.1)*	134	*(-6.9)*
Wk 13	132	137	*(+3.8)*	134	*(+1.5)*	132	*(±0.0)*	122	*(-7.6)*
Wk 28	151	154	*(+2.0)*	143	*(-5.3)*	142	*(-6.0)*	137	*(-9.3)*
Wk 36	156	151	*(-3.2)*	148	*(-5.1)*	148	*(-5.1)*	139	*(-10.9)*
Wk 52	150	148	*(-1.3)*	145	*(-3.3)*	138	*(-8.0)*	134	*(-10.7)*
Wk 1-52	7952	8083	*(+1.6)*	7854	*(-1.2)*	7700	*(-3.2)*	7290	*(-8.3)*
Recovery phase animals									
Wk 4	159	169	*(+6.3)*	161	*(+1.3)*	158	*(-0.6)*	147	*(-7.5)*
Wk 8	172	179	*(+4.1)*	175	*(+1.7)*	164	*(-4.7)*	159	*(-7.6)*
Wk 12	151	153	*(+1.3)*	154	*(+2.0)*	144	*(-4.6)*	143	*(-5.3)*
Wk 1-12	1928	2004	*(+3.9)*	1960	*(+1.7)*	1864	*(-3.3)*	1796	*(-6.8)*

	Dose level (ppm)							
	0	50		200		750		2,500	
Carcinogenicity phase animals									
Wk 1	142	143	*(+0.7)*	141	*(-0.7)*	142	*(±0.0)*	132	*(-7.0)*
Wk 13	144	139	*(-3.5)*	139	*(-3.5)*	142	*(-1.4)*	134	*(-6.9)*
Wk 28	150	144	*(-4.0)*	143	*(-4.7)*	145	*(-3.3)*	143	*(-4.7)*
Wk 36	149	145	*(-2.7)*	145	*(-2.7)*	154	*(+3.4)*	149	*(±0.0)*
Wk 52	161	152	*(-6.0)*	150	*(-6.8)*	155	*(-3.7)*	146	*(-9.3)*
Wk 64	164	165	*(+0.6)*	155	*(-5.5)*	167	*(+1.8)*	156	*(-4.9)*
Wk 76	173	159	*(-8.1)*	160	*(-7.5)*	169	*(-2.3)*	158	*(-8.7)*
Wk 96	171	176	*(+2.9)*	169	*(-1.2)*	173	*(+1.2)*	158	*(-7.6)*
Wk 104	161	150	*(-6.8)*	142	*(-11.8)*	145	*(-9.9)*	155	*(-3.7)*
Wk 1-104	16791	16547	*(-1.5)*	15937	*(-5.1)*	16441	*(-2.1)*	15886	*(-5.4)*

Wk: Week
Statistical analyses were not performed for food consumption data.

Low food conversion efficiencies were recorded during Week 1 in animals receiving 2,500 ppm and in females receiving 200 or 750 ppm. The overall food conversion efficiencies during the first 16 weeks of treatment (week 1-16) were low in females receiving 2,500 ppm (-13% and -12% in the Toxicity phase and Carcinogenicity phase females, respectively).

The overall food conversion efficiencies (week 1-16) of animals receiving up to 750 ppm were similar to those of the controls.

Table 5.5- 15 gives an overview of the food conversion efficiencies.

Table 5.5- 15: Food conversion efficiency in % *(% difference to control)*

	Dose level (ppm)								
	0	50		200		750		2,500	
Males									
Toxicity phase									
Week 1	28.7	28.1	*(-2.1)*	27.3	*(-4.9)*	25.9	*(-9.8)*	20.3	*(-29.3)*
Week 8	8.5	9.9	*(+16.5)*	9.0	*(+5.9)*	9.3	*(+9.4)*	9.6	*(+12.9)*
Week 16	4.9	4.4	*(-9.1)*	5.6	*(+14.3)*	5.3	*(+8.2)*	5.8	*(+18.4)*
Weeks 1-16	11.9	11.9	*(±0.0)*	11.9	*(±0.0)*	11.6	*(-2.5)*	11.3	*(-5.0)*
Carcinogenicity phase									
Week 1	25.4	25.8	*(+1.6)*	26.9	*(+5.9)*	24.8	*(-2.4)*	20.9	*(-17.7)*
Week 8	7.8	8.2	*(+5.1)*	7.9	*(+1.3)*	8.3	*(-6.4)*	9.1	*(+16.7)*
Week 16	4.6	5.0	*(+8.7)*	5.9	*(+28.3)*	6.1	*(+32.6)*	6.6	*(+43.5)*
Weeks 1-16	12.1	12.1	*(±0.0)*	12.2	*(+0.8)*	11.8	*(-2.5)*	11.4	*(-5.8)*
Females									
Toxicity phase									
Week 1	19.6	22.2	*(+13.3)*	12.0	*(-38.8)*	11.3	*(-42.3)*	9.5	*(-51.5)*
Week 8	4.0	5.3	*(+32.5)*	4.0	*(±0.0)*	4.5	*(+12.5)*	4.6	*(+15.0)*
Week 16	4.7	3.8	*(-19.1)*	2.4	*(-48.9)*	3.3	*(-29.8)*	3.4	*(-27.7)*
Weeks 1-16	6.7	6.6	*(-1.5)*	6.4	*(-4.5)*	6.3	*(-6.0)*	5.8	*(-13.4)*

	Dose level (ppm)								
	0	50		200		750		2,500	
Carcinogenicity phase									
Week 1	17.7	16.2	*(-8.5)*	14.0	*(-20.9)*	12.0	*(-32.2)*	13.7	*(-22.6)*
Week 8	4.3	5.2	*(+20.9)*	4.8	*(+11.6)*	4.0	*(-7.0)*	5.3	*(+23.3)*
Week 16	2.6	2.1	*(-19.2)*	2.5	*(-3.8)*	3.9	*(+50.0)*	3.4	*(+30.8)*
Weeks 1-16	6.9	6.5	*(-5.8)*	6.8	*(-1.4)*	6.7	*(-2.9)*	6.1	*(-11.6)*

Statistical analyses were not performed for food conversion data.

The overall achieved doses in mg/kg bw/day were as follows.

Table 5.5- 16: Overall achieved doses (mg/kg bw/day)

	Dose level (ppm)									
	Males					Females				
	0	50	200	750	2,500	0	50	200	750	2,500
Toxicity phase										
Week 1-52	-	2.5	9.8	37.0	125.5	-	3.3	12.9	48.7	163.6
Carcinogenicity phase										
Week 1-104	-	2.1	8.4	31.5	109.4	-	2.8	10.8	41.0	142.2

4. Ophthalmoscopic examinations

During Week 51 (Toxicity phase), 78 and 104 (Carcinogenicity phase) of treatment, there were no ophthalmoscopic abnormalities detected which were considered to be a result of treatment with fluopicolide.

5. Laboratory investigations

Haematology:

Changes seen in the blood during the study were confined, in general, to animals receiving 2,500 ppm, with males being affected more consistently than the females.

When compared with the controls, low haemoglobin concentrations were recorded throughout the treatment period in males and females receiving 2,500 ppm, except in Week 52 in males and Week 26 in females. Associated with this was a reduction of haematocrit (packed cell volumes) in males in Week 26 and 78 and in females in Week 13, 78 and 104 and low erythrocyte counts in females in Week 13 and 78. An initial reduction of haematocrit, haemoglobin concentration and erythrocyte count was evident in Week 13 in females receiving 750 ppm. No similar effect was observed subsequently, and these changes were, therefore, considered to be of no toxicological significance. As a result of these inter-group differences there were several changes in the calculated red blood cell parameters in both sexes at 2,500 ppm. Mean cell haemoglobin was consistently low in males and mean cell haemoglobin concentration was low on all occasions in males, with the exception of Week 26. The slightly low mean cell haemoglobin concentrations in Week 13, 26 and 52 and in females receiving 2,500 ppm were not attributed to treatment since the difference from controls was small and there was no other effect upon erythrocytes at these examinations. Mean cell volumes were low in Week 26, 52 and 104 in males.

Inter-group differences in erythrocytic parameters at the lower dietary concentrations of fluopicolide were minor and less consistent and, in view of this, were not considered toxicologically significant. These included slightly low mean cell haemoglobin concentrations in Week 13, 52 and 78 in males receiving 750 ppm, slightly low mean cell haemoglobin concentrations in Week 26 in females receiving 750 ppm and marginally low mean cell haemoglobin concentrations in Week 13 and 52 in males at 200 ppm.

None of these changes was evident at the end of the recovery period following 52-weeks of treatment.

At 50 ppm there were no treatment-related findings in the peripheral blood recorded for either sex.

All other inter-group differences that attained statistical significance were minor, lacked dosage-relationship or were inconsistent between examinations. Such changes included inter-group differences in clotting times in animals receiving 2,500 ppm, where activated partial thromboplastin times were high in Week 26 in males and low in females, whilst in Week 104 prothrombin times were shortened in males. They also included the occasional variations of lymphocyte count in females receiving 750 or 2,500 ppm since these were evident only in Weeks 13, 52 and 104. In view of the absence of these findings at Week 26 and 78 they could not, with any confidence, be attributed to treatment.

Examination of the tail vein smears did not indicate any treatment-related abnormalities in either sex. Statistically significant changes occurred in females receiving 2,500 ppm where lymphocyte counts were marginally low and eosinophil counts were slightly high in Week 52, whilst in Week 104 neutrophil counts were low and lymphocyte counts were high in males. Since these differences were not reflected in animals sampled at the same time for the main haematological examination, it is considered that these minor changes arose by chance and are not related to treatment.

Table 5.5- 17 gives an overview of the most relevant findings.

Table 5.5- 17: Haematological results *(% difference to control)*

Parameter	Dose level (ppm) 0	50		200		750		2,500	
	Males								
Week 13									
Hct (L/L)	0.446	0.451	*(+1.1)*	0.456	*(+2.2)*	0.445	*(-0.2)*	0.437	*(-2.0)*
Hb (g/dL)	15.3	15.4	*(+0.7)*	15.4	*(+0.7)*	15.1	*(-1.3)*	14.7**	*(-3.9)*
RBC (x10^{12}/L)	8.17	8.35	*(+2.2)*	8.38	*(+2.6)*	8.24	*(+0.9)*	8.24	*(+0.9)*
MCH (pg)	18.5	18.4	*(-0.5)*	18.5	*(±0.0)*	18.3	*(-1.1)*	17.9**	*(-3.2)*
MCHC (g/dL)	34.3	34.1	*(-0.6)*	33.9**	*(-1.2)*	33.9**	*(-1.2)*	33.7**	*(-1.7)*
MCV (fL)	54.0	54.0	*(±0.0)*	54.5	*(+0.9)*	54.1	*(+0.2)*	53.1	*(-1.7)*
Week 26									
Hct (L/L)	0.459	0.468	*(+1.9)*	0.465	*(+1.3)*	0.460	*(+0.2)*	0.442*	*(-3.7)*
Hb (g/dL)	15.4	15.7	*(+1.9)*	15.6	*(+1.3)*	15.5	*(+0.6)*	14.8**	*(-3.9)*
RBC (x10^{12}/L)	8.57	8.72	*(+1.8)*	8.67	*(+1.2)*	8.65	*(+0.9)*	8.50	*(-0.8)*
MCH (pg)	18.0	18.0	*(±0.0)*	18.0	*(±0.0)*	17.9	*(-0.6)*	17.5*	*(-2.8)*
MCHC (g/dL)	33.6	33.6	*(±0.0)*	33.5	*(-0.3)*	33.6	*(±0.0)*	33.5	*(-0.3)*
MCV (fL)	53.7	53.7	*(±0.0)*	53.7	*(±0.0)*	53.3	*(-0.7)*	52.1**	*(-3.0)*

Parameter	Dose level (ppm)									
	0	50		200		750		2,500		
Week 52										
Hct (L/L)	0.437	0.441	*(+0.9)*	0.451	*(+3.2)*	0.442	*(+1.1)*	0.428	*(-2.1)*	
Hb (g/dL)	15.0	15.1	*(+0.6)*	15.3	*(+2.0)*	15.2	*(+1.3)*	14.6	*(-2.7)*	
RBC ($x10^{12}$/L)	8.26	8.20	*(-0.7)*	8.45	*(+2.3)*	8.48	*(+2.7)*	8.41	*(+1.8)*	
MCH (pg)	18.2	18.6	*(+2.2)*	18.2	*(±0.0)*	17.9	*(-1.6)*	17.4**	*(-4.4)*	
MCHC (g/dL)	34.4	34.3	*(-0.3)*	34.0*	*(-1.2)*	34.2	*(-0.3)*	34.1*	*(-0.9)*	
MCV (fL)	52.9	54.2	*(+2.5)*	53.5	*(+1.1)*	52.2	*(-1.3)*	51.0**	*(-3.6)*	
Week 13 of recovery										
Hct (L/L)	0.423	0.425	*(+0.5)*	0.423	*(±0.0)*	0.439	*(+3.8)*	0.412	*(-2.6)*	
Hb (g/dL)	14.9	15.3	*(+2.7)*	15.3	*(+2.7)*	15.4	*(+3.4)*	14.7	*(-1.3)*	
RBC ($x10^{12}$/L)	8.17	8.10	*(-0.9)*	8.22	*(+0.6)*	8.15	*(-0.2)*	7.89	*(-3.4)*	
MCH (pg)	18.3	18.9	*(+3.3)*	18.6	*(+1.6)*	19.0	*(+3.8)*	18.6	*(+1.6)*	
MCHC (g/dL)	35.1	36.0**	*(+2.6)*	35.4	*(+0.9)*	35.2	*(+0.3)*	35.7*	*(+1.7)*	
MCV (fL)	52.1	52.5	*(+0.8)*	52.5	*(+0.8)*	53.9	*(+3.5)*	52.2	*(+0.2)*	
Week 78 (Carcinogenicity phase)										
Hct (L/L)	0.416	0.403	*(-3.1)*	0.425	*(+2.2)*	0.427	*(+2.6)*	0.389*	*(-6.5)*	
Hb (g/dL)	15.0	14.5	*(-3.3)*	15.2	*(+1.3)*	15.2	*(+1.3)*	13.8**	*(-8.0)*	
RBC ($x10^{12}$/L)	8.09	7.94	*(-1.9)*	8.43	*(+4.2)*	8.24	*(+1.9)*	7.80	*(-3.6)*	
MCH (pg)	18.6	18.2	*(-2.2)*	18.0	*(-3.2)*	18.4	*(-1.1)*	17.7**	*(-4.8)*	
MCHC (g/dL)	36.0	35.9	*(-0.3)*	35.7	*(-0.8)*	35.5**	*(-1.4)*	35.4**	*(-1.7)*	
MCV (fL)	51.5	50.7	*(-1.6)*	50.5	*(-1.9)*	51.9	*(+0.8)*	50.0	*(-2.9)*	
Week 104 (Carcinogenicity phase)										
Hct (L/L)	0.425	0.425	*(±0.0)*	0.439	*(+3.3)*	0.420	*(-1.2)*	0.409	*(-3.8)*	
Hb (g/dL)	14.4	14.4	*(±0.0)*	14.9	*(+3.5)*	14.1	*(-2.1)*	13.6	*(-5.6)*	
RBC ($x10^{12}$/L)	7.68	7.71	*(+0.4)*	7.98	*(+3.9)*	7.38	*(-3.9)*	7.68	*(±0.0)*	
MCH (pg)	18.8	18.8	*(±0.0)*	18.7	*(-0.5)*	19.2	*(+2.1)*	17.7**	*(-4.8)*	
MCHC (g/dL)	33.8	33.9	*(+0.3)*	33.9	*(+0.3)*	33.4	*(-1.2)*	33.2**	*(-1.8)*	
MCV (fL)	55.6	55.3	*(-0.5)*	55.0	*(-1.1)*	57.5	*(+3.4)*	53.2*	*(-4.3)*	
Females										
Week 13										
Hct (L/L)	0.439	0.438	*(-0.2)*	0.438	*(-0.2)*	0.429*	*(-2.3)*	0.426*	*(-3.0)*	
Hb (g/dL)	15.2	15.1	*(-0.7)*	15.2	*(±0.0)*	14.8**	*(-2.6)*	14.6**	*(-3.9)*	
RBC ($x10^{12}$/L)	7.92	7.80	*(-1.5)*	7.85	*(-0.9)*	7.63*	*(-3.7)*	7.68*	*(-3.0)*	
MCH (pg)	19.2	19.3	*(+0.5)*	19.3	*(+0.5)*	19.4	*(+1.0)*	19.0	*(-0.5)*	
MCHC (g/dL)	34.7	34.5	*(-0.6)*	34.6	*(-0.3)*	34.5	*(-0.6)*	34.3**	*(-1.2)*	
MCV (fL)	55.5	56.1	*(+1.1)*	55.8	*(+0.5)*	56.2	*(+1.3)*	55.5	*(0.0)*	

Parameter	Dose level (ppm)							
	0	50		200		750		2,500	
Week 26									
Hct (L/L)	0.423	0.428	*(+1.2)*	0.427	*(+0.9)*	0.427	*(+0.9)*	0.416	*(-1.7)*
Hb (g/dL)	14.8	14.8	*(±0.0)*	14.8	*(±0.0)*	14.7	*(-0.7)*	14.4	*(-2.7)*
RBC ($x10^{12}$/L)	7.64	7.57	*(-0.9)*	7.62	*(-0.3)*	7.59	*(-0.7)*	7.50	*(-1.8)*
MCH (pg)	19.3	19.6	*(+1.6)*	19.4	*(+0.5)*	19.4	*(+0.5)*	19.2	*(-0.5)*
MCHC (g/dL)	34.9	34.7	*(-0.6)*	34.6	*(-0.9)*	34.5	*(-1.4)*	34.5*	*(-1.4)*
MCV (fL)	55.4	56.5	*(+2.0)*	56.1	*(+1.3)*	56.3	*(+1.6)*	55.3	*(-0.2)*
Week 52									
Hct (L/L)	0.422	0.415	*(-1.7)*	0.418	*(-0.9)*	0.417	*(-1.2)*	0.409	*(-3.1)*
Hb (g/dL)	14.7	14.3	*(-2.7)*	14.5	*(-1.4)*	14.4	*(-2.0)*	14.0*	*(-4.8)*
RBC ($x10^{12}$/L)	7.44	7.24	*(-2.7)*	7.36	*(-1.1)*	7.30	*(-1.9)*	7.23	*(-2.8)*
MCH (pg)	19.8	19.8	*(±0.0)*	19.6	*(-1.0)*	19.7	*(-0.5)*	19.4	*(-2.0)*
MCHC (g/dL)	34.8	34.6	*(-0.6)*	34.6	*(-0.6)*	34.5	*(-0.9)*	34.2**	*(-1.7)*
MCV (fL)	56.8	57.3	*(+0.9)*	56.7	*(-0.2)*	57.2	*(+0.7)*	56.6	*(-0.4)*
Week 13 of recovery									
Hct (L/L)	0.403	0.397	*(-1.5)*	0.399	*(-1.0)*	0.403	*(±0.0)*	0.414	*(+2.7)*
Hb (g/dL)	14.4	14.2	*(-1.4)*	14.3	*(-0.7)*	14.3	*(-0.7)*	14.6	*(+1.4)*
RBC ($x10^{12}$/L)	7.25	7.16	*(-1.2)*	7.03	*(-3.7)*	6.96	*(-4.0)*	7.30	*(+0.7)*
MCH (pg)	19.9	19.8	*(-0.5)*	20.4	*(+2.5)*	20.5	*(+3.0)*	20.1	*(+1.0)*
MCHC (g/dL)	35.8	35.7	*(-0.3)*	35.9	*(+0.3)*	35.5	*(+0.8)*	35.4*	*(-1.1)*
MCV (fL)	55.7	55.5	*(-0.4)*	56.8	*(+2.0)*	57.8	*(+3.8)*	56.8	*(+2.0)*
Week 78 (Carcinogenicity phase)									
Hct (L/L)	0.417	0.400	*(-4.1)*	0.406	*(-2.6)*	0.406	*(-2.6)*	0.395*	*(-5.3)*
Hb (g/dL)	14.6	14.0	*(-4.1)*	14.4	*(-1.4)*	14.3	*(-2.1)*	13.9**	*(-4.8)*
RBC ($x10^{12}$/L)	7.39	7.03	*(-4.9)*	7.26	*(-1.8)*	7.17	*(-3.0)*	6.96**	*(-6.0)*
MCH (pg)	19.7	20.0	*(+1.5)*	19.9	*(+1.0)*	19.9	*(+1.0)*	19.9	*(+1.0)*
MCHC (g/dL)	35.3	35.1	*(-0.6)*	35.5	*(+0.6)*	35.2	*(-0.3)*	35.1	*(-0.6)*
MCV (fL)	55.8	57.0	*(+2.2)*	55.9	*(+0.2)*	56.6	*(+1.4)*	56.9	*(+2.0)*
Week 104 (Carcinogenicity phase)									
Hct (L/L)	0.404	0.408	*(+1.0)*	0.415	*(+2.7)*	0.399	*(-1.2)*	0.382*	*(-5.4)*
Hb (g/dL)	14.0	14.2	*(+1.4)*	14.4	*(+2.9)*	13.8	*(-1.4)*	13.1*	*(-6.4)*
RBC ($x10^{12}$/L)	6.97	7.09	*(+1.7)*	7.14	*(+2.4)*	6.87	*(-1.4)*	6.62	*(-5.0)*
MCH (pg)	20.1	20.1	*(±0.0)*	20.2	*(+0.5)*	20.2	*(+0.5)*	20.0	*(-0.5)*
MCHC (g/dL)	34.6	34.8	*(+0.6)*	34.7	*(+0.3)*	34.7	*(+0.3)*	34.4	*(-0.6)*
MCV (fL)	58.1	57.7	*(-0.7)*	58.2	*(+0.2)*	58.1	*(±0.0)*	58.1	*(±0.0)*

* $p \leq 0.05$, ** $p \leq 0.01$, significantly different from controls

Clinical chemistry:

Biochemical examination of the blood plasma at Week 13, 26 and 52 indicated, when compared with the controls, high total protein concentrations and low albumin to globulin ratios in males and females receiving 2,500 ppm. Males receiving 200 or 750 ppm had high total protein concentrations in Week 13 and 26 which, on the latter occasion, were associated with low albumin to globulin ratio.

There was no consistent effect upon plasma albumin concentration, with an increase being recorded only on Week 13 in males receiving 2,500 ppm. None of these differences was evident at the end of the recovery period, nor were they present at subsequent examinations during the remainder of the treatment period.

Males receiving 2,500 ppm had high plasma creatinine concentrations, compared with the controls, in Week 13, 26, 78 and 104, but not in Week 52, and there was no evidence for this trend being present at the end of the recovery period.

There was a trend throughout the treatment period towards high total plasma cholesterol concentrations in males receiving 2,500 ppm. This was observed at all investigations, with the exception of that performed in Week 78, and was also evident in Week 26 and 104 in males receiving 750 ppm. This was not present at the end of the 13-week recovery period. Statistical significance was only attained when compared with the controls, at the Week 13 investigation.

There were some disturbances of plasma electrolyte concentrations on many of the sampling occasions. Plasma potassium concentrations were high in Week 52 and 104 in males receiving 750 ppm and in males and females receiving 2,500 ppm, and in Week 52 in females receiving 750 ppm. Plasma calcium concentrations were high in Week 52 and 104 in males and females receiving 2,500 ppm, with animals receiving 750 ppm also being similarly affected in Week 104. These changes were not apparent at the end of the recovery period. All other inter-group differences in electrolyte levels were minor or lacked dosage-relationship and were, therefore, not considered toxicologically significant.

All other inter-group differences that attained statistical significance when compared with the controls were minor, lacked dosage-relationship or were not observed consistently during the treatment period. These differences were, therefore, not clearly attributable to treatment and are likely to represent normal biological variation.

Table 5.5-18: Selected clinical chemistry results *(% difference to control)*

Parameter	Dose level (ppm)							
	0	50		200		750		2,500	
Males								
Week 13								
Total protein (g/L)	64	64	*(±0.0)*	66*	*(+3.1)*	66*	*(+3.1)*	68**	*(+6.2)*
A/G ratio	1.15	1.15	*(±0.0)*	1.13	*(+1.7)*	1.10	*(-4.3)*	1.08*	*(-6.1)*
Cholesterol (mmol/L)	1.82	1.80	*(-1.1)*	1.90	*(+4.4)*	1.98	*(+8.8)*	2.14*	*(+17.6)*
Calcium (nmol/L)	2.70	2.74	*(+1.5)*	2.69	*(+0.4)*	2.74	*(+1.5)*	2.77	*(+2.6)*
Potassium (mmol/L)	4.5	4.3	*(-4.4)*	4.3	*(-4.4)*	4.2	*(-6.7)*	4.3	*(-4.4)*
Creatinine (µmol/L)	45	47	*(+4.4)*	49	*(+8.9)*	47	*(+4.4)*	50*	*(+11.1)*
Albumin (g/L)	34	34	*(±0.0)*	35	*(+2.9)*	35	*(+2.9)*	35**	*(+2.9)*

Parameter	Dose level (ppm)							
	0	50		200		750		2,500	
Week 26									
Total protein (g/L)	66	68	*(+3.0)*	68*	*(+3.0)*	68*	*(+3.0)*	68*	*(+3.0)*
A/G ratio	1.08	1.10	*(+1.9)*	1.03*	*(-4.6)*	1.02*	*(-5.6)*	1.04*	*(-3.7)*
Cholesterol (mmol/L)	1.82	1.57	*(-13.7)*	1.94	*(+6.6)*	2.07	*(+13.7)*	2.06	*(+13.2)*
Calcium (nmol/L)	2.80	2.81	*(+0.4)*	2.85	*(+1.8)*	2.82	*(+0.7)*	2.79	*(-0.4)*
Potassium (mmol/L)	4.1	4.1	*(±0.0)*	4.1	*(±0.0)*	4.1	*(±0.0)*	4.2	*(+2.4)*
Creatinine (µmol/L)	45	47	*(+4.4)*	44	*(-2.2)*	46	*(+2.2)*	50**	*(+11.1)*
Albumin (g/L)	34	34	*(±0.0)*	35	*(+2.9)*	34	*(±0.0)*	35	*(+2.9)*
Week 52									
Total protein (g/L)	69	67	*(-2.9)*	68	*(-1.4)*	69	*(±0.0)*	71*	*(+2.9)*
A/G ratio	0.97	1.01	*(+4.1)*	0.96	*(-1.0)*	0.95	*(-2.1)*	0.93	*(-4.1)*
Cholesterol (mmol/L)	2.46	2.30	*(-6.5)*	2.28	*(-7.3)*	2.66	*(+8.1)*	2.80	*(+13.8)*
Calcium (nmol/L)	2.83	2.84	*(+0.4)*	2.85	*(+0.7)*	2.88	*(+1.8)*	2.93**	*(+19.1)*
Potassium (mmol/L)	3.9	4.0	*(+2.6)*	4.0	*(+2.6)*	4.3**	*(+10.3)*	4.5**	*(+15.4)*
Creatinine (µmol/L)	50	50	*(±0.0)*	48	*(-4.0)*	50	*(±0.0)*	52	*(+4.0)*
Albumin (g/L)	34	34	*(±0.0)*	33	*(-2.9)*	34	*(±0.0)*	34	*(±0.0)*
Week 13 of recovery									
Total protein (g/L)	70	70	*(±0.0)*	69	*(-1.4)*	69	*(-1.4)*	69	*(-1.4)*
A/G ratio	2.34	2.64	*(+12.8)*	2.40	*(+2.6)*	2.89	*(+23.5)*	2.46	*(+5.1)*
Cholesterol (mmol/L)	2.90	2.89	*(-0.3)*	2.91	*(+0.3)*	2.90	*(±0.0)*	2.85	*(-1.7)*
Calcium (nmol/L)	3.8	3.8	*(±0.0)*	4.0	*(+5.3)*	3.9	*(+2.6)*	4.1**	*(+7.9)*
Potassium (mmol/L)	50	46	*(-8.0)*	46	*(-8.0)*	46	*(-8.0)*	50	*(±0.0)*
Creatinine (µmol/L)	35	34	*(-2.9)*	34	*(-2.9)*	34	*(-2.9)*	34	*(-2.9)*
Albumin (g/L)	0.98	0.96	*(-2.0)*	0.98	*(±0.0)*	0.96	*(-2.0)*	0.99	*(+1.0)*

Parameter	Dose level (ppm)							
	0	50		200		750		2,500	
Week 78 (Carcinogenicity phase)									
Total protein (g/L)	71	68	*(-4.2)*	69	*(-2.8)*	71	*(±0.0)*	73	*(+2.8)*
A/G ratio	0.97	0.97	*(±0.0)*	0.96	*(-1.0)*	0.95	*(-2.1)*	0.96	*(-1.0)*
Cholesterol (mmol/L)	3.18	2.51	*(-21.1)*	3.66	*(+15.1)*	3.53	*(+11.0)*	3.52	*(+10.7)*
Calcium (nmol/L)	2.97	2.93	*(-1.3)*	2.93	*(-1.3)*	3.01	*(+1.3)*	3.04	*(+2.4)*
Potassium (mmol/L)	4.0	4.0	*(±0.0)*	4.0	*(±0.0)*	4.2	*(+10.0)*	4.2	*(+10.0)*
Creatinine (µmol/L)	46	45	*(-2.2)*	47	*(+2.2)*	48	*(+4.3)*	50*	*(+8.7)*
Albumin (g/L)	35	33	*(-5.7)*	34	*(-2.9)*	35	*(±0.0)*	35	*(±0.0)*
Week 104 (Carcinogenicity phase)									
Total protein (g/L)	71	69	*(-2.8)*	71	*(±0.0)*	71	*(±0.0)*	73	*(+2.8)*
A/G ratio	0.91	0.87	*(-4.4)*	0.92	*(+1.1)*	0.88	*(-3.3)*	0.85	*(-6.6)*
Cholesterol (mmol/L)	3.34	3.24	*(-3.0)*	4.08	*(+22.2)*	4.49	*(+34.4)*	4.39	*(+31.4)*
Calcium (nmol/L)	2.85	2.88	*(+1.1)*	2.87	*(+0.7)*	2.95*	*(+3.5)*	3.07**	*(+7.7)*
Potassium (mmol/L)	3.5	3.6	*(+2.9)*	3.6	*(+2.9)*	4.1**	*(+17.1)*	4.0**	*(+11.4)*
Creatinine (µmol/L)	48	50	*(+4.2)*	50	*(+4.2)*	52	*(+8.3)*	58**	*(+20.8)*
Albumin (g/L)	34	32	*(-5.9)*	34	*(±0.0)*	33	*(-2.9)*	33	*(-2.9)*
Females									
Week 13									
Total protein (g/L)	70	69	*(-1.4)*	68	*(-2.9)*	70	*(±0.0)*	74*	*(-5.7)*
A/G ratio	1.20	1.17	*(-2.5)*	1.15	*(-4.2)*	1.17	*(-2.5)*	1.10*	*(-8.3)*
Cholesterol (mmol/L)	2.35	2.02	*(-14.0)*	2.24	*(-4.7)*	2.44	*(+3.8)*	2.44	*(+3.8)*
Calcium (nmol/L)	2.81	2.79	*(-0.7)*	2.78	*(-1.1)*	2.83	*(+0.7)*	2.88	*(+2.5)*
Potassium (mmol/L)	3.8	3.8	*(±0.0)*	3.9	*(+2.6)*	3.6	*(-5.3)*	3.8	*(±0.0)*
Creatinine (µmol/L)	50	52	*(+4.0)*	51	*(+2.0)*	50	*(±0.0)*	52	*(+4.0)*
Albumin (g/L)	38	37	*(-2.6)*	36	*(-5.3)*	38	*(±0.0)*	39	*(+2.6)*

Parameter	Dose level (ppm)									
	0	50		200		750		2,500		
Week 26										
Total protein (g/L)	71	72	*(+1.4)*	73	*(+2.8)*	73	*(+2.8)*	79**	*(+11.3)*	
A/G ratio	1.20	1.21	*(+0.8)*	1.15	*(-4.2)*	1.14	*(-5.0)*	1.07*	*(-10.8)*	
Cholesterol (mmol/L)	2.55	2.14	*(-16.1)*	2.72	*(+6.7)*	2.58	*(+1.2)*	2.81	*(+10.2)*	
Calcium (nmol/L)	2.89	2.83	*(-2.1)*	2.90	*(+0.3)*	2.90	*(+0.3)*	2.98	*(+3.1)*	
Potassium (mmol/L)	3.8	3.7	*(-2.6)*	3.7	*(+2.6)*	4.0	*(+5.3)*	3.9	*(+2.6)*	
Creatinine (µmol/L)	49	52	*(+6.1)*	50	*(+2.0)*	47	*(-4.1)*	50	*(+2.0)*	
Albumin (g/L)	39	39	*(±0.0)*	39	*(±0.0)*	39	*(±0.0)*	41	*(+5.1)*	
Week 52										
Total protein (g/L)	77	78	*(+1.3)*	79	*(+2.6)*	77	*(±0.0)*	82**	*(+6.5)*	
A/G ratio	1.12	1.16	*(+3.6)*	1.08	*(-3.6)*	1.12	*(±0.0)*	1.01**	*(-9.8)*	
Cholesterol (mmol/L)	3.16	2.94	*(-7.0)*	3.30	*(+4.4)*	3.38	*(+7.0)*	3.60	*(+13.9)*	
Calcium (nmol/L)	2.93	2.90	*(-1.0)*	2.97	*(+1.4)*	2.96	*(+1.0)*	3.00*	*(+2.4)*	
Potassium (mmol/L)	3.1	2.9	*(-6.5)*	3.2	*(+3.2)*	3.6**	*(+16.1)*	3.6**	*(+16.1)*	
Creatinine (µmol/L)	56	59	*(+5.4)*	54	*(-3.7)*	54	*(-3.6)*	58	*(+3.6)*	
Albumin (g/L)	41	42	*(+2.4)*	41	*(±0.0)*	41	*(±0.0)*	41	*(±0.0)*	
Week 13 of recovery										
Total protein (g/L)	73	75	*(+2.7)*	75	*(+2.7)*	75	*(+2.7)*	73	*(±0.0)*	
A/G ratio	1.14	1.14	*(±0.0)*	1.14	*(±0.0)*	1.14	*(±0.0)*	1.10	*(-3.5)*	
Cholesterol (mmol/L)	2.59	2.85	*(+10.0)*	2.96	*(+14.3)*	2.67	*(+3.1)*	2.60	*(+0.4)*	
Calcium (nmol/L)	2.88	2.93	*(+1.7)*	2.88	*(±0.0)*	2.88	*(±0.0)*	2.83	*(-1.7)*	
Potassium (mmol/L)	3.5	3.5	*(±0.0)*	3.5	*(±0.0)*	3.5	*(±0.0)*	3.6	*(+2.9)*	
Creatinine (µmol/L)	54	53	*(+3.9)*	53	*(+3.9)*	54	*(+5.9)*	55	*(+7.8)*	
Albumin (g/L)	39	40	*(+2.6)*	40	*(+2.6)*	40	*(+2.6)*	38	*(-2.6)*	

Parameter	Dose level (ppm)									
	0	50		200		750		2,500		
Week 78 (Carcinogenicity phase)										
Total protein (g/L)	74	73	*(-1.4)*	76	*(+2.7)*	74	*(±0.0)*	77	*(+4.1)*	
A/G ratio	1.13	1.09	*(-3.5)*	1.08	*(-4.4)*	1.14	*(+0.9)*	1.10	*(-2.6)*	
Cholesterol (mmol/L)	3.42	2.76	*(-19.3)*	3.08	*(-9.9)*	3.35	*(-2.0)*	3.26	*(-4.7)*	
Calcium (nmol/L)	2.88	2.81	*(-2.4)*	2.82	*(-2.1)*	2.85	*(-1.0)*	2.90	*(+0.7)*	
Potassium (mmol/L)	3.5	3.6	*(+2.9)*	3.3	*(-5.7)*	3.6	*(+2.9)*	3.6	*(+2.9)*	
Creatinine (µmol/L)	55	56	*(+1.8)*	50	*(-9.1)*	50	*(-9.1)*	53	*(-3.6)*	
Albumin (g/L)	39	38	*(-2.6)*	39	*(±0.0)*	40	*(+2.6)*	40	*(+2.6)*	
Week 104 (Carcinogenicity phase)										
Total protein (g/L)	76	77	*(+1.3)*	76	*(±0.0)*	75	*(-1.3)*	80	*(+5.3)*	
A/G ratio	1.03	1.01	*(-1.9)*	1.04	*(+1.0)*	1.07	*(+3.9)*	0.97	*(-5.8)*	
Cholesterol (mmol/L)	3.53	3.52	*(-0.3)*	3.20	*(-9.3)*	3.61	*(+2.3)*	4.15	*(+22.7)*	
Calcium (nmol/L)	2.77	2.83	*(+2.2)*	2.87*	*(+3.6)*	2.96**	*(+6.9)*	2.95**	*(+6.5)*	
Potassium (mmol/L)	3.3	3.1	*(-6.1)*	3.2	*(-3.0)*	3.6	*(+9.1)*	3.9*	*(+18.2)*	
Creatinine (µmol/L)	49	48	*(-2.0)*	47	*(-4.1)*	45	*(-8.2)*	49	*(±0.0)*	
Albumin (g/L)	38	38	*(±0.0)*	39	*(+2.6)*	39	*(+2.6)*	39	*(+2.6)*	

* $p \leq 0.05$; ** $p \leq 0.01$, statistically different to control using Fisher's Exact test

Urinalysis:

There was no clear effect of treatment upon urinary composition. There were occasional variations in urinary composition during the treatment period, but they were minor, lacked dosage-relationship or were seen on only one occasion. In view of the absence of a consistent difference in any of the measured urinalysis parameters, the differences that were observed are considered to represent normal biological variation.

6. Sacrifice and pathology

Necropsy:

Macroscopic examination of the toxicity phase animals killed after 52 weeks of treatment or after 13 weeks of recovery revealed no findings related to the administration of fluopicolide in the diet.

In the carcinogenicity phase animals, which were killed or dying during the treatment period, lower incidences of mammary masses were reported in females that had received 200, 750 or 2,500 ppm.

In Carcinogenicity phase animals killed after 104 weeks of treatment there were several findings attributed to treatment in males that had received 2,500 ppm. These findings included a high incidence of skin masses (see histopathology chapter for further discussion) and a low incidence of scabs on the tail. When all animals of the carcinogenicity phase were considered together and comparisons made with the controls, slightly high incidences of enlarged kidneys and thyroids and a low incidence of scabs on the tail were evident in males which had received 2,500 ppm. In females given 2,500 ppm there was a lower incidence of adrenal masses whilst in those given 200, 750 or 2,500 ppm there were lower incidences of mammary masses which match with the clinical observation that in females the number of animals with swellings and the total number of swellings were lower in treated groups than in the controls. However, this finding is not considered an adverse effect.

Organ weights:

Evaluation of the organ weight data indicated increased relative kidney weights after 52 and 104 weeks of treatment in males given 750 or 2,500 ppm, with females given 2,500 ppm being similarly affected after 52 weeks of treatment only. In addition, there was a statistically significant increase in absolute kidney weights in males treated ≥ 200 ppm after 104 weeks; however, the effect at the 200 ppm is considered an artefact secondary to the increased body weight compared to controls (+6%) at this dose level (relative kidney weight was comparable to control). This is supported by the absence of histopathological kidney effects at 200 ppm.

Absolute and relative liver weights were increased after 52 and 104 weeks of treatment in males given 2,500 ppm, with relative liver weights also being higher after 52 weeks in males given 750 ppm and in females given 2,500 ppm compared to control. None of the changes apparent after 52 weeks of treatment were present after completion of the 13-week recovery period, indicating that full recovery had occurred.

After 104 weeks of treatment there was a slight increase of the absolute and bodyweight-relative thyroid weight, compared with the controls, in males given 2,500 ppm. This was not evident at the interim kill after completion of 52 weeks of treatment.

Several other organ weight difference attained statistical significance when compared with the controls but these were confined to one sex, not dosage-related or, in females given 2,500 ppm, considered to be associated with low bodyweight gains. Consequently, they are considered to represent normal biological variation.

The organ weights of both sexes given 50 or 200 ppm and females at 750 ppm were not affected by treatment.

Table 5.5- 19 gives an overview of selected organ weight results.

Table 5.5- 19: Selected organ weights after 52 weeks of treatment *(% difference to control)*

	Dose level (ppm)									
	0	**50**		**200**		**750**		**2,500**	
Males										
Terminal body weight	740.2	735.3	*(-0.7)*	738.9	*(-0.2)*	727.7	*(-1.7)*	711.6	*(-3.9)*
Kidney										
Absolute weight (g)	3.87	4.00	*(+3.4)*	3.98	*(+2.8)*	4.13	*(+6.7)*	4.20	*(+8.5)*
Relative weight to body weight (%)	0.526	0.546	*(+3.8)*	0.541	*(+2.9)*	0.571*	*(+8.6)*	0.592**	*(+12.5)*
Liver										
Absolute weight (g)	21.51	22.17	*(+3.1)*	22.86	*(+6.3)*	23.17	*(+7.7)*	24.81*	*(+15.3)*
Relative weight to body weight (%)	2.917	3.015	*(+3.4)*	3.075	*(+5.4)*	3.183*	*(+9.1)*	3.491**	*(+19.7)*
Thyroid (+ para)										
Absolute weight (g)	0.029	0.027	*(-6.9)*	0.028	*(-3.4)*	0.033	*(+13.8)*	0.029	*(±0.0)*
Relative weight to body weight (%)	0.0039	0.0037	*(-5.1)*	0.0038	*(-2.6)*	0.0046*	*(+17.9)*	0.0041	*(+5.1)*
Females										
Terminal body weight	464.1	427.9	*(-7.8)*	438.8	*(-3.9)*	413.9	*(-10.8)*	386.0**	*(-16.8)*
Kidney										
Absolute weight (g)	2.78	2.60	*(-6.5)*	2.72	*(-2.2)*	2.64	*(-5.0)*	2.67	*(-4.0)*
Relative weight to body weight (%)	0.604	0.617	*(+2.2)*	0.620	*(+2.9)*	0.643	*(+6.5)*	0.694**	*(+14.9)*
Liver										
Absolute weight (g)	14.47	14.07	*(-2.8)*	14.51	*(+0.3)*	13.94	*(-3.7)*	14.31	*(-1.1)*
Relative weight to body weight (%)	3.130	3.307	*(+5.7)*	3.319	*(+6.0)*	3.384	*(+8.1)*	3.719**	*(+18.8)*
Thyroid (+ para)										
Absolute weight (g)	0.023	0.022	*(-4.3)*	0.022	*(-4.3)*	0.022	*(-4.3)*	0.023	*(±0.0)*
Relative weight to body weight (%)	0.0050	0.0051	*(+2.0)*	0.0051	*(+2.0)*	0.0053	*(+6.0)*	0.0058	*(+16.0)*

* p ≤ 0.05, ** p ≤ 0.01, statistically different to control using Fisher's Exact test

Table 5.5- 20: Selected organ weights after 104 weeks of treatment *(% difference to control)*

	Dose level (ppm)							
	0	**50**		**200**		**750**		**2500**	
Males									
Terminal body weight	810.7	820.6	*(+1.2)*	856.6	*(+5.7)*	791.8	*(-2.3)*	744.9	*(-8.1)*
Kidney									
Absolute weight (g)	4.67	4.83	*(+3.4)*	5.10*	*(+9.2)*	5.08*	*(+8.8)*	5.69**	*(+21.8)*
Relative weight to body weight (%)	0.586	0.592	*(+1.0)*	0.603	*(+2.9)*	0.655*	*(+11.8)*	0.800**	*(+36.5)*
Liver									
Absolute weight (g)	22.49	23.03	*(+2.4)*	24.18	*(+7.5)*	24.21	*(+7.6)*	27.42**	*(+21.9)*
Relative weight to body weight (%)	2.803	2.843	*(+1.4)*	2.850	*(+1.7)*	3.100	*(+10.6)*	3.757**	*(+34.0)*
Thyroid (+ para)									
Absolute weight (g)	0.039	0.041	*(+5.1)*	0.047	*(+20.5)*	0.041	*(+5.1)*	0.060*	*(+53.8)*
Relative weight to body weight (%)	0.0049	0.0050	*(+2.0)*	0.0055	*(+12.2)*	0.0053	*(+8.2)*	0.0080*	*(+63.3)*
Adrenals									
Absolute weight (g)	0.373	0.084	*(-77.5)*	0.084	*(-77.5)*	0.152	*(-59.2)*	0.081	*(-78.3)*
Relative weight to body weight (%)	0.0467	0.0103	*(-77.9)*	0.0098	*(-79)*	0.0218	*(-53)*	0.0115	*(-75.4)*
Females									
Terminal body weight	511.1	504.3	*(-1.3)*	540.2	*(+5.7)*	554.6	*(+8.5)*	484.1	*(-5.3)*
Kidney									
Absolute weight (g)	3.41	3.39	*(-0.6)*	3.18	*(-6.7)*	3.32	*(-2.6)*	3.23	*(-5.3)*
Relative weight to body weight (%)	0.641	0.700	*(+9.2)*	0.613	*(-4.4)*	0.631	*(-1.6)*	0.694	*(+8.3)*
Liver									
Absolute weight (g)	18.97	17.22	*(-9.2)*	16.79	*(-11.5)*	18.64	*(-1.7)*	17.28	*(-8.9)*
Relative weight to body weight (%)	3.541	3.456	*(-2.4)*	3.148	*(-11.1)*	3.440	*(-2.9)*	3.657	*(+3.3)*
Thyroid (+ para)									
Absolute weight (g)	0.034	0.029	*(-14.7)*	0.032	*(-5.9)*	0.035	*(+2.9)*	0.032	*(-5.9)*
Relative weight to body weight (%)	0.0063	0.0056	*(-11.1)*	0.0060	*(-4.8)*	0.0065	*(+3.2)*	0.0067	*(+6.3)*
Adrenals									
Absolute weight (g)	0.174	0.130	*(-25.3)*	0.191	*(+9.8)*	0.124	*(-28.7)*	0.092**	*(-47.1)*
Relative weight to body weight (%)	0.0334	0.0274	*(-18)*	0.0313	*(-6.3)*	0.0231	*(-30.8)*	0.0202	*(-39.5)*

* $p \leq 0.05$; ** $p \leq 0.01$; statistically different to control using Fisher's Exact test

7. Histopathology

Animals killed after 52 weeks of treatment:

Findings considered related to treatment were seen in the liver and kidneys. There were no neoplastic findings considered related to treatment with fluopicolide.

An increased incidence of centrilobular hepatocytic hypertrophy was observed in males given fluopicolide at 750 and 2,500 ppm.

Table 5.5- 21: Incidences of liver findings after 52 weeks in males – Number of animals affected

Findings	Dose level (ppm)				
	0	50	200	750	2,500
Liver					
Centrilobular hypertrophy	0/20	0/20	0/15	14/20***	19/20***

* p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001, statistically different to control using Fisher's Exact-test

An increased incidence and/or severity of cortical tubular basophilia was observed in the kidneys of males given fluopicolide at 750 or 2,500 ppm. This was associated with increased incidences of other degenerative changes in the kidneys, as shown in Table 5.5- 22. There was an increased incidence of hyperplasia of the papillary epithelium in the kidneys of female animals receiving 200 ppm or above (see Table 5.5- 22). This finding occurred at a minimal or slight level and was usually associated with mineralisation of the papillary/pelvic epithelium, the incidence of which did not show a similar relationship to treatment.

Table 5.5- 22: Selected kidney findings after 52 weeks – Number of animals affected

Findings	Dose level (ppm)				
	0	50	200	750	2,500
Males					
Cortical tubular basophilia	7/20	10/20	9/15	20/20***	20/20***
Cortical tubules with hyaline droplets	0/20	1/20	0/15	2/20	13/20***
Granular medullary casts	0/20	0/20	0/15	0/20	7/20**
Hyaline tubular casts	6/20	5/20	4/15	8/20	17/20**
Females					
Hyperplasia, papillary epithelium	2/16	3/18	8/17	7/18	9/19*

* p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001, statistically different to control using Fisher's Exact test

Findings indirectly related to treatment included a reduced incidence and severity of chronic myocarditis in the heart of males given fluopicolide at 2,500 ppm. This is a common age-related degenerative change and the reduced incidence of this finding is considered to be secondary to the treatment-related body weight changes observed in these animals. As such, it is considered a non adverse finding.

Reduced incidences of a number of findings achieved statistical significance compared with control animals. These findings comprised reduced incidences of periportal hepatocyte vacuolation in the liver, sinus erythrocytes/erythrophagocytosis in the mandibular lymph nodes, dilated glands in the stomach and thymic haemorrhage. It is doubtful that any of these findings are related to treatment, and even if reduced incidences of these findings are not considered indicative of an adverse effect.

Other findings were of a type and severity commonly seen in rats of this age at this laboratory.

Animals killed after 13 weeks of the recovery period:

The majority of changes reported as related to treatment at the end of the 52-week treatment period were no longer apparent at the end of the 13-week recovery period. Only a slight increase in the severity of cortical tubular basophilia in the kidneys of males was still apparent, as shown in Table 5.5- 23.

Table 5.5- 23: Incidences of kidney findings after 13-week recovery in males – Number of animals affected

Findings	Dose level (ppm)				
	0	**50**	**200**	**750**	**2,500**
Number examined	9	9	10	10	9
Kidney					
Cortical tubular basophilia					
Minimal	3	3	6	1	2
Slight	5	5	4	7	4
Moderate	0	1	0	2	3
Total incidence	8	9	10	10	9

Carcinogenicity phase:

Neoplastic findings

There were no neoplastic findings that were considered related to treatment with fluopicolide, especially no liver adenomas or carcinomas. The statistical analysis of the tumours in carcinogenicity phase animals similarly did not detect any statistically significant differences.

A non-statistically significant increase in the incidence of benign skin tumours was recorded in male rats given fluopicolide at 2,500 ppm (see Table 5.5- 24). The incidence was above the reported historical control data range given in a supplementary document ([redacted] 2005; M-263575-01-1). Unexpectedly, the control and lower dose incidences were also clearly above the presented historical control data range. The animal number per group was generally 60 animals, however referring to the numbers given in the study report only for 24, 28, 25, 20 and 34 animals the skin was histopathologically examined in the control and the different treatment groups. However, in the pathology part of the material and methods section in the study report it is described that "Tissues reported at macroscopic examination as being grossly abnormal were examined for all animals" and that "The absence of a comment for a tissue scheduled for examination therefore indicates that the tissue was examined and found to be normal". Therefore, it is assumed that all 60 animals per group were examined for skin tumours, but only for animals with lesions a full histopathologic examination of the affected area was performed (see revised Table 5.5- 26). After recalculation most incidences of the control and lower treatment groups are within the reported HCD ranges and the tumour incidence for males at 2,500 ppm is 13.3% compared to a HCD range of 0-10.8% for keratoacanthoma and 8.3% compared to a HCD range of 0-3.1% for squamous cell papilloma. This approach is supported by the statement of the study report author considering the incidence of these tumours as only slightly higher than the background incidence in rats of this strain in this laboratory.

Skin tumours are relatively common tumours that are often associated with previous damage to the skin, and since there was no statistical significance, no clear-dose dependency at the lower dose levels and only males were affected, the slightly higher incidences seen at 2,500 ppm are considered to have arisen by chance and are not considered to be of any toxicological importance in the oncogenic assessment of fluopicolide.

Table 5.5- 24: Incidences of skin tumours in carcinogenicity phase animals *(%)*

Findings	Dose level (ppm)					
	0	50	200	750	2,500	HCD[#]
Males						
Keratoacanthoma	3/24 *(12.5)*	3/28 *(10.7)*	5/25 *(20)*	3/26 *(11.5)*	8/34 *(23.5)*	0-10.8%
Squamous cell papilloma	2/24 *(8.3)*	0/28 *(0.0)*	1/25 *(4.0)*	2/26 *(7.7)*	5/34 *(14.7)*	0-3.1%
Females						
Keratoacanthoma	1/20 *(5.0)*	0/15 *(0.0)*	0/13 *(0.0)*	0/10 *(0.0)*	0/13 *(0.0)*	0-1.7%
Squamous cell papilloma	1/20 *(5.0)*	0/15 *(0.0)*	0/13 *(0.0)*	0/10 *(0.0)*	0/13 *(0.0)*	0-1.5%

[#] Range of percentages of nine chronic or carcinogenesis studies performed between 1998/99 in the same lab and CD rats of the same supplier for a study duration of 104 weeks ([redacted] 2005; M-263575-01-1).

Table 5.5- 25: Incidences of skin tumours in carcinogenicity phase animals (%) - revised

Findings	Dose level (ppm)					
	0	50	200	750	2,500	HCD[#]
Males						
Keratoacanthoma	3/60 *(5.0)*	3/60 *(5.0)*	5/60 *(8.3)*	3/60 *(5.0)*	8/60 *(13.3)*	0-10.8%
Squamous cell papilloma	2/60 *(3.3)*	0/60 *(0.0)*	1/60 *(1.7)*	2/60 *(3.3)*	5/60 *(8.3)*	0-3.1%
Females						
Keratoacanthoma	1/60 *(1.7)*	0/60 *(0.0)*	0/60 *(0.0)*	0/60 *(0.0)*	0/60 *(0.0)*	0-1.7%
Squamous cell papilloma	1/60 *(1.7)*	0/60 *(0.0)*	0/60 *(0.0)*	0/60 *(0.0)*	0/60 *(0.0)*	0-1.5%

[#] Range of percentages of nine chronic or carcinogenesis studies performed between 1998/99 in the same lab and CD rats of the same supplier for a study duration of 104 weeks ([redacted] 2005; M-263575-01-1).

Non-neoplastic findings

Findings considered related to treatment were seen in the liver, kidneys, pancreas and prostate.

An increased incidence of centrilobular hepatocytic hypertrophy was observed in males given fluopicolide at 200 ppm or above. An increased incidence and/or severity of cystic degeneration and foci of alteration was seen in male rats and an increased incidence of eosinophilic foci of alteration in females given 750 or 2,500 ppm (see Table 5.5- 26).

Table 5.5- 26: Incidences of liver findings of carcinogenicity phase animals – Number of animals affected

Findings	Dose level (ppm)									
	Males					Females				
	0	50	200	750	2,500	0	50	200	750	2,500
Non neoplastic findings										
Number examined	60	60	60	60	60	60	60	60	60	60
Hepatocyte hypertrophy	0	0	2	9**	18***	0	0	0	0	0
Cystic degeneration	13	17	18	23	32***	0	1	0	0	0
Clear cell foci	22	29	28	36*	35*	21	12	20	17	23
Eosinophilic foci	13	17	15	19	25*	4	6	5	11	15*

* p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001, statistically different to control

An increased incidence and/or severity of a number of degenerative and proliferative changes were observed in the kidneys of males and females (see

Table 5.5- 27). The majority of these changes were limited to male animals given 2,500 ppm. The occurrence of hyperplasia of the papillary epithelium showed an increased incidence and/or severity in females given 750 ppm or above. This finding was usually associated with mineralisation of the papillary/pelvic epithelium. There was no clear relationship to treatment in the incidence or severity of these findings in males.

Table 5.5- 27: Incidence and severity of kidney findings of carcinogenicity phase animals

Findings	Dose level (ppm)									
	Males					Females				
	0	50	200	750	2,500	0	50	200	750	2,500
Number examined	60	60	60	60	60	60	60	60	60	60
Tubular casts										
Minimal	10	15	19	19	10	14	11	12	11	16
Slight	13	14	12	9	27	5	4	6	11	4
Moderate	1	1	1	4	8	4	2	2	2	5
Total incidence	24	30	32	32	45***	23	17	20	24	25
Pelvic calculi										
Minimal	2	0	0	0	0	2	1	0	1	0
Slight	3	2	5	5	2	3	4	1	2	5
Moderate	1	0	2	3	6	0	0	0	1	3
Total incidence	6	2	7	8	8	5	5	1	4	8
Cortical tubular basophilia										
Minimal	5	12	6	12	3	15	11	18	17	16
Slight	32	22	32	30	22	36	36	33	32	24
Moderate	10	13	15	10	22	5	3	6	7	13
Marked	1	3	1	1	1	0	1	0	0	1
Total incidence	48	50	54	53	48	56	51	57	56	54

Findings	Dose level (ppm)									
	Males					Females				
	0	50	200	750	2,500	0	50	200	750	2,500
Cortical tubular dilation										
Minimal	3	2	1	3	6	3	2	4	6	0
Slight	6	5	7	3	17	4	4	4	5	12
Moderate	1	2	1	2	3	1	1	1	1	2
Marked	0	0	0	0	1	0	0	0	0	0
Total incidence	10	9	9	8	27**	8	7	9	12	14
Cortical tubules with hyaline droplets										
Minimal	2	1	0	2	4	0	0	0	0	0
Slight	0	1	1	2	7	1	0	0	1	0
Moderate	0	1	2	0	1	0	2	0	0	0
Marked	1	0	0	0	2	0	0	0	0	0
Total incidence	3	3	3	4	14**	1	2	0	1	0
Hyperplasia, papillary epithelium										
Minimal	4	6	7	6	3	20	17	15	23	16
Slight	10	8	8	12	6	15	7	14	17	17
Moderate	0	0	3	2	1	1	4	3	7	10
Marked	0	0	0	0	1	0	0	0	0	0
Total incidence	14	14	18	20	11	36	28	32	47*	43
Interstitial inflammation										
Minimal	3	4	4	5	3	1	0	0	1	0
Slight	9	3	9	10	15	2	4	2	5	11
Moderate	2	1	1	2	5	1	1	1	0	0
Total incidence	14	8	14	17	23	4	5	3	6	11
Cortical cysts										
Minimal	1	1	1	2	1	2	1	0	0	0
Slight	1	0	3	4	4	1	3	1	1	2
Moderate	1	1	1	3	5	0	0	0	1	0
Marked	0	0	1	0	1	0	0	0	0	0
Total incidence	3	2	6	9	11*	3	4	1	2	2
Mineralization papillary/pelvic epithelium										
Minimal	11	3	4	5	4	27	27	27	27	21
Slight	4	0	3	1	1	12	9	14	18	15
Moderate	0	0	1	0	1	1	4	1	4	8
Total incidence	15	3**	8	6	6	40	40	42	49	44
Mineralization papilla										
Minimal	9	1	0	2	7	4	3	2	0	3
Slight	0	0	2	0	5	0	0	0	0	0
Total incidence	0	1	2	2	12***	4	3	2	0	3

* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$, statistically different to control

An increased incidence and/or severity of atrophic changes was observed in the pancreas of animals given 2,500 ppm (see Table 5.5- 28), evident in males as acinar atrophy and in females as acinar replacement by adipose tissue. In the adrenals, the incidences of focal hypertrophy (cortical with vacuolation and zonal glomerulosa) was statistically significantly decreased in females at the high dose.

Table 5.5- 28: Incidences of pancreas and adrenal findings of carcinogenicity phase animals

Findings	Dose level (ppm)									
	Males					Females				
	0	50	200	750	2,500	0	50	200	750	2,500
Pancreas										
Pancreas, number examined	60	33	33	32	60	60	45	38	37	59
Acinar atrophy	17	13	12	19	26	8	2	8	12	11
Acinar replacement by adipose tissues	14	6	4	7	16	6	3	0	6	16*
Adrenals										
Adrenals, number examined	60	47	45	46	60	60	57	54	54	60
Focal cortical hypertrophy with vacuolation	14	10	8	6	9	29	20	18	23	12**
Zona glomerulosa – focal hypertrophy	18	12	13	10	14	17	14	10	13	6*

* $p \leq 0.05$, **$p \leq 0.01$, statistically different to control

An increased incidence of acinar atrophy, often associated with reduced colloid, was present in the prostate of males at 2,500 ppm only and is considered secondary to the decreased body weight and stress at this dose level.

Table 5.5- 29: Incidences of prostate findings of carcinogenicity phase animals

Findings	Dose level (ppm)				
	0	50	200	750	2,500
Acinar cell atrophy	19/60	12/35	7/34	6/31	31*/60
Reduced colloid	8/60	3/35	7/34	3/31	17/60

* $p \leq 0.05$, statistically different to control

As findings indirectly related to treatment, an increased incidence of cystic follicular cell hyperplasia was present in the thyroids of males given fluopicolide at 750 ppm or above. This finding was considered to be secondary to the increased metabolic activity of the liver at these dose levels indicated by increased liver weights at ≥ 750 ppm and an increased incidence of centrilobular hepatocytic hypertrophy at ≥ 200 ppm in males. This is evidenced by a concomitant increase in liver weights from 750 ppm at 52 and 104 weeks and an increase in the incidence of centrilobular hypertrophy from 750 ppm at 52 weeks and from 200 ppm at 104 weeks. Each individual animal presenting with thyroid follicular cell adenoma or hyperplasia also presented with an increased liver weight, thus further strengthening the argument. Furthermore, mechanistic *in vitro* studies (mouse, rat and human hepatocytes and CAR/Ko & PXR/Ko mice) and *in vivo* studies (28-day in mice and 7-day study in rats at 2500 ppm) showed hepatic changes and enzyme induction profiles with a CAR/PXR dependent phenobarbital-like profile.

Table 5.5- 30: Incidences of thyroid findings of carcinogenicity phase animals

Findings	Dose level (ppm)									
	Males					Females				
	0	50	200	750	2,500	0	50	200	750	2,500
For animals killed or dying during the treatment period										
Thyroid, number examined	37	32	29	35	31	39	45	36	36	34
Cystic follicular cell hyperplasia	0	1	0	3	0	0	0	1	0	0
For animals killed after 104 weeks of treatment										
Thyroid, number examined	23	7	7	6	29	21	3	2	3	25
Cystic follicular cell hyperplasia	0	0	0	1	7*	1	0	0	0	1

* $p \leq 0.05$, statistically different to control

An increased incidence of sinus erythrocytosis/erythrophagocytosis, which attained statistical significance in the highest dietary concentration group, was seen in the mandibular lymph nodes of females. This is a relatively common finding in rats of all ages and is considered to have arisen by chance.

An increased incidence of interstitial cell hyperplasia of the testes of rats at 750 and 2,500 ppm was recorded, associated with a slightly increased incidence of benign interstitial (Leydig) cell adenoma. However, neither incidence attained statistical significance. Since the incidences of adenomas were additionally within the background range seen in this laboratory and there was no dose-response relationship the slightly increased adenoma incidences are considered incidental and reflective of the normal biological variability. Furthermore, no treatment-related effect on testes histopathology was observed in animals killed after 52 weeks.

Table 5.5- 31: Incidences of testes findings of carcinogenicity phase animals (%)

Findings	Dose level (ppm)					
	0	50	200	750	2,500	HCD[#]
Testes: Interstitial cell hyperplasia	6/60 (10%)	4/39 (10.3%)	3/38 (7.9%)	7/35 (20%)	14/60 (23.3%)	-
Benign interstitial (Leydig) cell adenoma	1/60 (1.6%)	2/39 (5.1%)	2/38 (5.3%)	3/35 (8.6%)	4/60 (6.7%)	0.0-9.2%

* $p \leq 0.05$, statistically different to control

[#] Range of percentages of nine chronic or carcinogenesis studies performed between 1998/99 in the same lab and CD rats of the same supplier for a study duration of 104 weeks ([redacted] 2005; M-263575-01-1).

A number of other findings showed statistically significant changes in incidence compared with the control groups in one or both of the sexes (mainly decreased incidences). These findings were generally of low incidence and lacked dosage-relationship and were, therefore, considered to be of no toxicological significance.

III. Conclusion

It is concluded that the administration of fluopicolide to CD rats at dietary concentrations of up to 2,500 ppm for 104 weeks did not provide any evidence of oncogenic potential. The liver and kidneys were identified as target organs. A small number of findings were recorded for animals receiving 200 ppm. However, these were considered non adverse, e.g. adaptive liver changes in 2/60 males. This dietary concentration is therefore considered the No Observed Adverse Effect Level (NOAEL) in this study, equivalent to 8.4 mg/kg bw/day in males and 10.8 mg/kg bw/day in females. No treatment-related changes were detected in animals receiving 50 ppm and this dietary concentration is considered the No Observed Effect Level (NOEL) in this study, equivalent to 2.1 mg/kg bw/day in males and 2.8 mg/kg bw/day in females.

Assessment and conclusion by applicant:

The study was conducted according to OECD TG 453 and is valid and acceptable to investigate the long-term toxicity and carcinogenic potential of fluopicolide in rats. Fluopicolide was not carcinogenic in rats. A NOAEL of 200 ppm (equivalent to 8.4 and 10.8 mg/kg bw/d in males and females) was determined from this study.

Data Point:	KCA 5.5/02
Report Author:	[redacted]
Report Year:	2003
Report Title:	AE C638206 - Carcinogenicity study by oral route (dietary admixutre) in C57BL/6 mice
Report No:	C038732
Document No:	M-225595-01-1
Guideline(s) followed in study:	EEC 96/54/EEC, B.32 (1996); JMAFF: 59 NohSan No. 4200 (1985); OECD: 451 (1981); US-EPA OPPTS 870.4200 (1985)
Deviations from current test guideline:	Methods: Deviations from current guideline SANCO/3029/99 rev. 4: There is no calibration plot or calibration equation presented, however the calibration range and coefficient of determination (>0.999) are reported. The accuracy and precision data are determined from dietary admixtures containing the test item rather than fortified samples. However, there are 6 samples per concentration level with mean recoveries between 70-110% and RSD < 20%. Considering that this analytical method is validated in support of a toxicological cold study, the method validation is considered fit for purpose. Study: Deviations from the current OECD guideline (451, 2018): - Coagulating glands were not sampled, fixed or examined histopathologically This minor deviation(s) is considered not to compromise the results and outcome of the study."
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Data Point:	KCA 5.5/03
Report Author:	[redacted]
Report Year:	2005
Report Title:	Historical control data for long-term studies in C57bl/6 mice
Report No:	IRF.24.10.05
Document No:	M-263691-01-1
Guideline(s) followed in study:	in support of OPPTS 870.4200
Deviations from current test guideline:	Deviations from the current OECD guideline (451, 2018): - Coagulating glands were not sampled, fixed or examined histopathologically This minor deviation(s) is considered not to compromise the results and outcome of the study.
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

The objective of this study was to evaluate the potential carcinogenicity of floupicolide (batch number: OP2050046, purity: 95.9% at study start) following daily oral administration (dietary admixture) to C57BL/6 mice for 78 weeks.

Four principal treated groups, each composed of 50 male and 50 female C57BL/6 mice received the test substance mixed with the diet at constant concentrations of 0 (control), 50, 400 or 3,200 ppm for 78 weeks. The concentrations of 50, 400 and 3,200 ppm corresponded on average to 7.9, 64.5 and 551.0 mg/kg bw/day for the males and to 11.5, 91.9 and 772.3 mg/kg bw/day for the females. For

evaluation of toxic effects, 10 satellite males and 10 satellite females per group were sacrificed after a one-year period of administration.

Throughout the study, clinical signs and mortality were checked daily, and careful examination was carried out before the beginning of the treatment period and weekly thereafter to assess possible neurotoxic effects.

Palpation of possible masses was carried out every 4 weeks from Weeks 4 to 52 and every 2 weeks thereafter. Body weight and food consumption were measured at weekly intervals during the first 13 weeks of the study, every 4 weeks until Weeks 31/32 and every 2 weeks thereafter. Achieved dosages were calculated. Before the sacrifice of satellite animals in Week 53, blood was taken for the determination of liver enzyme activities (aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase). At the end of the appropriate scheduled treatment period (52 or 78 weeks), animals were sacrificed and were submitted to a macroscopic post-mortem examination. A complete range of organs and any masses or macroscopic lesions were sampled. A microscopic examination was performed on all principal animals as well as on the liver and macroscopic abnormalities from the satellite animals (all groups).

The distribution of mortality, as well as the factors contributing to mortality or premature sacrifice, was similar in the control and treated groups. The incidence, nature and onset of the clinical signs were similar in the control and treated groups. No signs of neurotoxicity were observed during the study. The frequency, time of onset and size of the few palpable masses recorded were similar in the control and treated groups.

Food consumption and body weight of treated animals at 50 or 400 ppm were similar to that of controls.

When compared to the controls, the body weight and the body weight gain of the 3,200 ppm group was severely affected (body weight gain vs. controls in Week 78 vs. Week 1: males: -45%; females: -35%) by the treatment with the test substance. This effect correlated with a slight reduction of the food consumption.

There was no relevant difference in the liver enzyme activities between the treated and control groups.

Absolute and relative liver weights were higher in animals given 400 and 3,200 ppm at the end of 52-week and 78-week treatment periods. These changes were associated with hepatocellular hypertrophy noted among these animals.

After 52 weeks, liver enlargement was seen at 400 or 3,200 ppm in males only, and presence of masses and nodules in the liver in females treated at 3,200 ppm. After 78 weeks, a marked increase of liver enlargement was seen at 3,200 ppm, and the number of animals bearing masses and nodules in the liver in treated groups at 400 and 3,200 ppm was higher when compared with controls.

Microscopic examination at both 52- and 78-week revealed a dose-related hepatocellular hypertrophy at 400 ppm and 3,200 ppm, and a higher incidence of altered cell foci and hepatocellular adenoma at 3,200 ppm in both sexes at week 78.

In conclusion, as treatment-related effects, severe reductions of body weight gain and food consumption at 3,200 ppm were noted, thus indicating that the Maximal Tolerated Dose (MTD) was reached. Further observations were higher liver weights, enlarged liver, masses and nodules in the liver at 400 and 3,200 ppm, hepatocellular hypertrophy at 400 and 3,200 ppm, and high incidence of altered cell foci at 3,200 ppm and increased hepatocellular adenoma incidences at 3,200 ppm. This finding in the liver tissue might be attributed, at least in part, to the fact that 3,200 ppm reached the MTD. Additionally, mechanistic studies have demonstrated that the mode of action for the increased incidence of liver adenomas was found to be CAR-mediated and thus secondary to liver enzyme induction like that of phenobarbital (see [redacted] 2004; M-229594-01-1). This MoA is considered of no relevance in humans.

Therefore, under the experimental conditions, the No Observed Adverse Effect level (NOAEL) was 50 ppm (corresponding to 7.9 mg/kg bw/day for the males and 11.5 mg/kg bw/day for the females) for toxicity and 400 ppm (corresponding to 64.5 mg/kg bw/day for the males and 91.9 mg/kg bw/day for the females) for carcinogenicity.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 (fluopicolide)
Purity:	95.9%
Batch no.:	OP2050046

2. Vehicle and/or positive control

Vehicle:	Diet

3. Test animals

Species:	Mouse
Strain:	C57BL/6 Cr1:BR
Age:	7 weeks old
Weight at start:	23.3 g (range: 21.5 g to 26.2 g) for the males and 19.5 g (range: 17.7 g to 22.3 g) for the females.
Source:	[redacted]
Acclimation period:	Yes
Diet:	Powdered diet batch Nos. 1351, 1763 and 2373 (type: M20 EXTRALABO controlled, irradiated diet, supplier: SDS, 95420 Vigny, France)
Water:	Tap water (filtered with a 0.22 µm filter) contained in bottles
Housing:	The animals were individually housed in polycarbonate cages (24.0 x 13.5 x 13.0 cm) containing autoclaved sawdust (SICSA, Alfortville, France). The cages were placed in numerical order on the racks. On a monthly basis, all the racks were moved clockwise around the room, rack by rack
Temperature:	22± 2°C
Humidity:	50± 20%
Air changes:	Approximately 12 cycles/hour of filtered, non-recycled air
Photoperiod:	12 hours

B. Study design

1. In-life dates: April 19, 2001 to November 15, 2002

2. Animal assignment and treatment

The mice were randomized and assigned to the following test groups.

Table 5.5- 32: Study design

Group no.	Dose (ppm)	Number of males	Number of females
Chronic phase (53-week)			
1	0	10	10
2	50	10	10
3	400	10	10
4	3,200	10	10
Oncogenicity phase (78-80 weeks)			
1	0	50	50
2	50	50	50
3	400	50	50
4	3,200	50	50

The dose-levels were specified based on the results of a preliminary 90-day toxicity study in C57BL/6 mice ([redacted] 2006, M-205579-02-1). In this study, male and female C57BL/6 mice received 50, 200, 800 and 3200 ppm of fluopicolide in diet for at least 90 days. Body weight gains were slightly reduced (around -10%) at 3,200 ppm while higher liver weights associated with hepatocellular hypertrophy were observed in almost all the animals at 800 ppm and above. The NOEL was set at 50 ppm. Therefore, in the current carcinogenicity study, the dose-level of 3,200 ppm was selected as the highest dose-level which should produce signs of systemic toxicity without altering the normal life span of the animals. The dose-level of 50 ppm was selected as the lowest dose which should not produce any signs of toxicity and the dose-level of 400 ppm as the mid-range between the high and low dose-levels.

The oncogenicity phase animals were used for the evaluation of carcinogenic effects. The chronic phase animals were used to evaluate effects on target organs such as the liver after a one-year administration period.

The study duration was 53 weeks (i.e. 364 or 365 days) for the Chronic phase groups and 78 to 80 weeks (i.e. 546 to 557 days) according to the necropsy schedule for the Oncogenicity groups.

3. Diet preparation and analysis

The oral route was selected since it is a potential route of exposure in man. The test substance mixed with the diet was supplied to principal animals ad libitum for a period of 78 to 80 weeks (or 53 weeks for the satellite animals).

The HPLC analysis of the test substance carried out every 6 months confirmed that the purity remained the same throughout the treatment period.

The results of the analyses demonstrated the satisfactory homogeneity of each dietary admixture analysed during the study.

Furthermore, there was a good correspondence between the nominal and the measured concentrations of the test substance in the diet.

The results of the stability analyses of the dietary admixtures were summarized as follows:

Table 5.5- 33: Stability results

Concentration (ppm)	50	3,200
Open feeders (Animal room conditions)	Stable 10 days	Stable 16 days
Closed bags (room temperature)	Stable 13 days	Stable 35 days

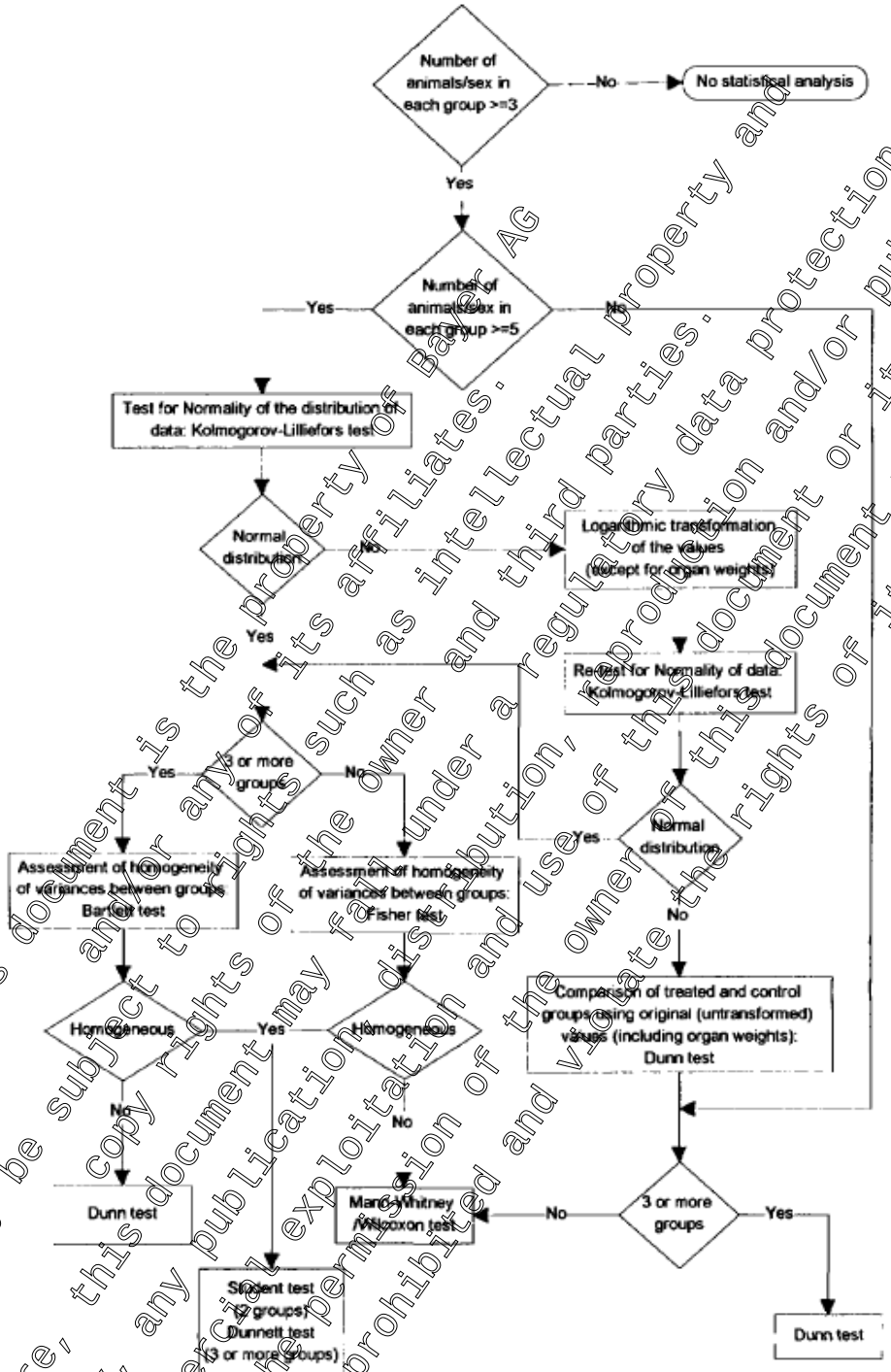
Throughout the study, a satisfactory agreement was observed between the nominal and actual concentrations of the test substance in the dietary admixtures administered since the deviations from nominal concentrations were in an acceptable range of ± 10%.

4. Statistics

Principal group animals and satellite animals were evaluated separately.

In vivo and organ weight data:

The following sequence was used for the statistical analyses of body weight, food consumption, haematology, blood biochemistry and organ weight data:



Analysis of survival and tumour data:

Survival rates were compared using the Chi-squared test.

The number of neoplasms (per group and per organ) were compared by Peto's test.

C. Methods

1. Observations

Each animal was checked at least twice a day (except on weekends and public holidays: at least once a day), for mortality or signs of morbidity. Any animal showing signs of poor clinical condition was humanely killed. When possible, a blood sample for the preparation of a blood smear was taken ante mortem for haematological examination. A macroscopic post-mortem examination was performed on all animals and the required tissues preserved for a microscopic examination.

Each animal was observed at least once a day, at approximately the same time, for the recording of clinical signs.

In addition to this routine check, a careful examination was carried out at least once a week. This observation included, but was not limited to, evaluation of skin and fur, eyes and mucous membranes, respiratory and circulatory effects, autonomic effects (such as salivation), central nervous system (including tremors and convulsions), changes in the level of activity, gait and posture, reactivity to handling or sensory stimuli, altered strength and stereotypies or bizarre behaviour (e.g. self-mutilation, walking backwards).

From Week 4, all animals were palpated every 4 weeks, and then, from Week 52, every 2 weeks for the remainder of the study in order to record the date of detection, location, size, appearance and progression of palpable masses.

2. Body weight

The body weight of each animal was recorded once before allocation of the animals to groups, on the first day of treatment, once a week during the first 13 weeks of the treatment period and then once every 4 weeks until Week 31. Since a markedly lower body weight gain was noted among the high-dose group, body weight was recorded every 2 weeks from Week 32 until the end of the study.

3. Food intake

The quantity of food consumed by the animals of each cage was recorded once a week, over a 7-day period, during the first 13 weeks of the treatment period and then every 4 weeks until Week 30. Since a markedly lower body weight gain was noted among the high-dose group, food consumption was recorded every 2 weeks from Week 31 until the end of the study. Food consumption was calculated per animal and per day.

4. Laboratory investigations

Oncogenicity animals:

In Week 52 and before the terminal sacrifice (Week 78), blood smears were prepared from samples obtained without anaesthesia from a tail vein of each surviving animal. When practicable, this was also done for any animal killed prematurely. The animals were not fasted before sampling.

After discussion with the Sponsor, it was considered that no specific signs of toxicity evidenced the need to determine the differential white cell count.

Chronic phase animals:

In Week 53, before sacrifice, and after an overnight fasting period, blood samples were collected under light isoflurane anaesthesia from the orbital sinus of each surviving male and female mouse of all animals.

The following parameters were determined:

Table 5.5- 34: Laboratory parameters

Alkaline phosphatase (ALP)	IU/L
Aspartate aminotransferase (ASAT)	IU/L
Alanine aminotransferase (ALAT)	IU/L

Bone marrow (oncogenicity and chronic phase animals):

Bone marrow smears were prepared from the femoral bone of all animals killed in Week 53 and at terminal sacrifice. After discussion with the Sponsor, it was considered that no specific signs of toxicity evidenced the need to determine the bone marrow differential cell count.

5. Sacrifice and pathology

On completion of the 52- or 78-week treatment periods, after at least 14 hours fasting, all surviving animals were asphyxiated by carbon dioxide and killed by exsanguination.

Any moribund animals were killed in the same way (except for fasting).

Macroscopic pathology:

A complete macroscopic post-mortem examination was performed on all animals, including any that died during the study or were killed prematurely. This included examination of the external surfaces, all orifices, the cranial cavity, the external surfaces of the brain and spinal cord, the thoracic, abdominal and pelvic cavities with their associated organs and tissues and the neck with its associated organs and tissues.

Organ weights:

At study termination, the body weight of all satellite and all principal group animals were recorded before necropsy, and the organs specified in the Tissue Procedures Table were weighed wet as soon as possible after dissection. Paired organs (i.e. epididymides, kidneys, ovaries and testes) were weighed together.

The following organ weights were determined.

Table 5.5- 35: Organ weights

Adrenals	Liver
Brain (including medulla/pons, cerebellar and cerebral cortex)	Ovaries
Epididymides	Spleen
Heart	Testes
Kidneys	Uterus with cervix

The ratio of organ weight to body weight (recorded immediately before sacrifice) was calculated.

Tissue sampling and fixation:

For all animals including any that died during the study or were killed prematurely, the tissues specified in Table 5.5- 36 were preserved in 10% buffered formalin (except for the eyes and Harderian glands which were fixed in Davidson's fixative, and the testes and epididymides which were preserved in Bouin's fluid).

Table 5.5- 36: Sampled organs and tissues

Organs	Preservation of tissue	Microscopic examination	Organs	Preservation of tissue	Microscopic examination
Macroscopic lesions	X	X	Ovaries	X	X
Adrenals	X	X	Pancreas	X	X
Aorta	X	X	Pharynx	X	X
Brain (including medulla/ pons cerebellar and cerebral cortex)	X	X	Pituitary gland	X	X
Cecum	X	X	Prostate	X	X
Colon	X	X	Rectum	X	X
Duodenum	X	X	Salivary glands (sublingual, submaxillary)	X	X
Epididymides	X	X	Sciatic nerve	X	X
Oesophagus	X	X	Seminal vesicles	X	X
Eyes (with Harderian glands)	X	X	Skeletal muscle	X	X
Femoral bone with articulation	X	X	Skin	X	X
Gall bladder	X	X	Spinal cord (cervical, thoracic, lumbar)	X	X
Heart	X	X	Spleen	X	X
Ileum	X	X	Sternum with bone marrow	X	X
Jejunum	X	X	Stomach with forestomach	X	X
Kidneys	X	X	Testes	X	X
Larynx	X	X	Thymus	X	X
Liver	X	X	Thyroids with parathyroid	X	X
Lungs with bronchi	X	X	Tongue	X	
Lymph nodes (mandibular and mesenteric)	X	X	Trachea	X	X
Mammary glands/area	X	X	Urinary bladder	X	X
Nose	X	X	Uterus (horns and cervix)	X	X
Optic nerve	X	X	Vagina	X	X

6. Histopathology

Tissue processing:

All tissues required for microscopic examination were embedded in paraffin wax, sectioned and stained with haematoxylin-eosin.

Microscopic examination:

All tissues required for microscopic examination were embedded in paraffin wax, sectioned at a thickness of approximately four microns and stained with haematoxylin-eosin.

A microscopic examination was performed on:

- all tissues listed in Table 5.5- 35 for all oncogenicity animals,
- all macroscopic lesions and liver of all chronic phase animals.

All the slides were sent to a pathologist for peer review of the following slides:

- all organs and tissues of 10% of the control and high dose-group animals,
- all tumors or neoplastic lesions,
- the liver of all animals

The microscopic findings presented in the report reflect the mutually agreed diagnoses.

II. Results and Discussion

A. Results

1. Mortality, clinical results

Mortality:

The number and occurrence of unscheduled deaths was summarized as follows:

Table 5.5-37: Mortality

Concentration (ppm)	0	50	400	3,200
Males				
- found dead	5	3	2	3
- prematurely killed	4	3	3	3
Total	9/50	6/50	5/50	6/50
Females				
- found dead	3	3	2	9
- prematurely killed	2	6	2	0
Total	5/50	9/50	4/50	9/50

The number and occurrence of unscheduled deaths was low and comparatively similar in all the control and treated groups, except for the females of the 3,200 ppm group in which mortality was slightly higher than in the control group. This was due to the higher number of animals found dead during the first 52 weeks of treatment. Taking into consideration that a comparable number of unscheduled deaths was noted in the low-dose group, a relationship to treatment with the test substance was considered to be improbable.

At study termination (Week 78), the survival rates were as follows:

Table 5.5- 38: Survival rate (%)

Concentration (ppm)	0	50	400	3,200
Males (week 1-78)	82	88	90	88
Females (week 1-78)	90	82	92	82

The survival rate was similar in the control and the treated groups. The differences observed between these groups were slight, not statistically significant and not dose-related.

The factors contributing to death or to premature killing were comparatively similar in treated and control groups, so that there was no treatment effect. The mean duration of treatment was similar in the control and treated groups.

Clinical signs:

The nature, incidence and onset of clinical signs were similar in the animals of both sexes from the control and treated groups. None of them suggested a treatment- or dose-relationship. Additionally they were among those commonly observed in mice. The most frequently recorded signs during the study were non-specific and/or related to the poor clinical condition of aged animals, before death or sacrifice.

No signs of neurotoxic effects were observed during the careful examination performed at weekly intervals.

The number of palpable masses recorded during the in vivo phase of the study did not indicate a dose- or treatment-relationship.

2. Body weights

At 50 and 400 ppm, body weight and body weight gain of treated animals were comparable to control values.

Animals treated at 3,200 ppm lost weight during the first week of the study (week 1-2: -0.2 g in males and -0.1 g in females compared to positive body weight gain of the other dose group animals), leading to statistically significant differences of body weight. After this period, overall body weight gain of animals treated at 3,200 ppm was severely reduced until the end of the dosing period (week 1-78: 45% in males and -35% in females).

Table 5.5- 39 gives an overview about the body weights and body weight gains.

Table 5.5- 39: Mean body weight and body weight gain

	Dose level (ppm)						
	0	**50**		**400**		**3,200**	
Males							
Body weight [g] *(% difference to control)*							
Week 1	23.1	23.0	*(-0.4)*	23.2	*(+0.4)*	23.3	*(+0.9)*
Week 2	23.9	23.9	*(±0.0)*	24.0	*(+0.4)*	23.1**	*(-3.3)*
Week 13	29.8	29.9	*(+0.3)*	29.0*	*(-2.7)*	26.9*	*(-9.7)*
Week 26	34.9	34.8	*(-0.3)*	33.8	*(-3.2)*	29.1**	*(-16.6)*
Week 52	40.8	41.3	*(+1.2)*	39.0	*(-4.4)*	32.9**	*(-21.8)*
Week 78	41.4	43.5	*(+5.1)*	42.0	*(+1.4)*	33.3**	*(-19.6)*
Body weight gain [g] *(% difference to control)*							
Week 1-2	0.8	0.9	*(+12.5)*	0.8	*(±0.0)*	-0.2	*(-125.0)*
Week 2-13	5.9	6.0	*(+1.7)*	5.0**	*(-15.3)*	3.9**	*(-33.9)*
Week 13-26	5.1	4.9	*(-3.9)*	4.8	*(-5.9)*	2.2**	*(-56.9)*
Week 26-52	5.8	6.4	*(+10.3)*	5.3	*(-8.6)*	2.8**	*(-51.7)*
Week 52-78	0.6	1.9	*(+216.7)*	3.0**	*(+400.0)*	1.1	*(+83.3)*
Week 1-78	18.3	20.5	*(+12.0)*	18.8	*(+2.7)*	10.0**	*(-45.4)*
Females							
Body weight [g] *(% difference to control)*							
Week 1	19.4	19.3	*(-0.5)*	19.4	*(±0.0)*	19.3	*(-0.5)*
Week 2	20.3	20.4	*(+0.5)*	20.3	*(±0.0)*	19.2**	*(-5.4)*
Week 13	24.5	24.5	*(±0.0)*	24.8	*(+1.2)*	22.8**	*(-6.9)*
Week 26	28.1	28.8	*(+2.5)*	28.7	*(+2.1)*	24.5**	*(-12.8)*
Week 52	33.3	34.5	*(+3.6)*	34.1	*(+2.4)*	26.7**	*(-19.8)*
Week 78	34.7	36.1	*(+4.0)*	36.3	*(+4.6)*	29.2**	*(-15.9)*
Body weight gain [g] *(% difference to control)*							
Week 1-2	0.9	1.1	*(+22.2)*	0.9	*(±0.0)*	-0.1**	*(-111.1)*
Week 2-13	4.2	4.1	*(-2.4)*	4.5	*(+7.1)*	3.6*	*(-14.3)*
Week 13-26	3.6	4.3	*(+19.4)*	3.9	*(+8.3)*	1.7**	*(-52.8)*
Week 26-52	5.2	5.7	*(+9.6)*	5.4	*(+3.8)*	2.2**	*(-57.7)*
Week 52-78	1.4	1.6	*(+14.3)*	2.2	*(+57.2)*	2.5	*(+78.6)*
Week 1-78	18.3	16.8	*(-9.8)*	16.9	*(+10.5)*	9.9**	*(-35.3)*

* $p \leq 0.05$; ** $p \leq 0.01$ statistically different to control

3. Food and test substance intake

Food consumption was similar in the control and the 50 or 400 ppm groups throughout the study. There was a tendency to lower food consumption in animals treated at 3,200 ppm, and mean values of the high-dose group were frequently statistically different from controls. These lower food consumption values correlated with an effect on body weight (see Table 5.5- 39).

Group mean food consumption values (g/animal/day) are summarized in 5.5- 40.

Table 5.5- 40: **Mean food consumption in g/animal/day** *(% difference to control)*

	Dose level (ppm)						
	0	50		400		3,200	
Males							
Week 1-12	5.8	5.7	*(-1.7)*	5.7	*(-1.7)*	5.6**	*(-3.4)*
Week 13-26	5.4	5.6	*(+3.7)*	5.5	*(+1.9)*	4.9**	*(-9.3)*
Week 29-51	5.4	5.5	*(+1.9)*	5.4	*(±0.0)*	4.8**	*(-11.1)*
Week 53-77	5.0	5.1	*(+2.0)*	5.0	*(±0.0)*	4.6**	*(-8.0)*
Week 1-78	5.4	5.5	*(+1.9)*	5.4	*(±0.0)*	5.0**	*(-7.4)*
Females							
Week 1-12	7.8	7.7	*(-1.3)*	7.9	*(+1.3)*	6.7**	*(-14.1)*
Week 13-26	6.7	6.9	*(+3.0)*	6.9	*(+3.0)*	6.1**	*(-9.0)*
Week 29-51	6.0	6.1	*(+1.7)*	6.0	*(±0.0)*	5.9**	*(-1.7)*
Week 53-77	5.6	5.7	*(+1.8)*	5.5	*(-1.8)*	5.1**	*(-8.9)*
Week 1-78	6.4	6.5	*(+1.6)*	6.5	*(+1.6)*	5.9**	*(-7.8)*

** $p \leq 0.01$, statistically different to control

The achieved doses of the test item over the whole study period were 0, 7.9, 64.5 and 551 mg/kg bw/d in males and 0, 11.5, 91.9 and 772.3 mg/kg bw/d in females.

4. Laboratory investigations

When compared with the mean control values, increase in liver enzyme activities was recorded in the females treated with 3,200 ppm, with a high standard variation, however attaining statistical significance for ALP activity.

Taking into account that these differences were due to the contribution of 2/10 females only and in absence of relevant changes in males, it was concluded that there were no differences of biological importance between control and treated animals in the activity of liver enzymes.

At interim sacrifice in Week 53, measurements of the activities of alkaline phosphatase (ALP), aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) are summarized in Table 5.5- 41.

Table 5.5- 41: **Liver enzyme activities (mean, expressed as IU/L), n = 10** *(% difference to control)*

Parameter	Dose level (ppm)						
	0	50		400		3,200	
Males							
Alkaline phosphatase	129	122	*(-5.4)*	114*	*(-11.6)*	135	*(+4.7)*
Aspartate aminotransferase	80	61	*(-23.8)*	62	*(-22.5)*	65	*(-18.8)*
Alanine aminotransferase	28	24	*(-14.3)*	40	*(+42.9)*	64	*(+128.6)*
Females							
Alkaline phosphatase	180	170	*(-5.6)*	183	*(+1.7)*	564**	*(+213.3)*
Aspartate aminotransferase	75	101	*(+34.7)*	115	*(+53.3)*	194	*(+158.7)*
Alanine aminotransferase	45	29	*(-35.6)*	34	*(-24.4)*	145	*(+222.2)*

* $p \leq 0.05$; ** $p \leq 0.01$, statistically different to control

5. Sacrifice and pathology

Macroscopic post-mortem examination:

After 52 weeks, in the chronic phase groups, liver enlargement was noted in one male given 400 ppm and in two males given 3,200 ppm. Masses and nodules were noted in the liver of two females given 3,200 ppm. These necropsy findings were considered to be treatment-related and correlated with the microscopic changes noted in the liver of these animals (hepatocellular hypertrophy, see microscopic examination).

All the other necropsy findings were found with similar incidences in both control and treated animals and showed no indication of treatment- or dose-relationship.

After 78 weeks, there was a marked increase of liver enlargement at 3,200 ppm, and the number of animals bearing masses and nodules in the liver in treated groups at 400 and 3,200 ppm was higher when compared with controls (see Table 5.5- 42).

Table 5.5- 42: Incidence of liver enlargement and masses and nodules in liver (Week 78)

Findings	Dose level (ppm)							
	Males				Females			
	0	50	400	3,200	0	50	400	3,200
Number of animals examined	50	50	50	50	50	50	50	50
Liver enlargement	2	7	6	28	8	3	5	29
Liver masses and nodules	3	2	6	10	1	2	3	9

The above-mentioned necropsy findings were considered to be treatment-related and correlated with the hepatic histopathological findings (hepatocellular hypertrophy and hepatocellular adenomas).

Most of the other necropsy findings observed were recognized to be those commonly occurring in aging mice of this strain and age such as enlargement and/or cystic appearance of the lymph nodes, kidneys, ovaries and uterus; enlargement of the spleen and seminal vesicles; paleness of the kidneys and small testes.

All these changes were of similar incidence and severity in both control and treated animals and showed no indication of treatment or dose-relationship.

In addition, the masses and/or nodules were equally distributed among the groups and showed no indication of a treatment-relationship.

Organ weights:

The principal differences (in %) noted between treated and control animals after 52 and 78 weeks were the absolute and relative liver weights (see Table 5.5- 43).

Table 5.5- 43: **Mean absolute and relative liver weights after 52 & 72 weeks (g)** *(% difference to control)*

Parameter	Dose level (ppm)					
	0	50		400		3,200	
Males							
Week 52							
Final body weight (g)	37.66	37.90	*(+0.6)*	42.56	*(+13.0)*	31.25*	*(-17.0)*
Absolute liver weight (g)	1.59	1.71	*(+7.5)*	2.06**	*(+29.6)*	2.15**	*(+35.2)*
Relative liver weight (% of body weight)	4.21	4.53	*(+7.8)*	4.84	*(+15.0)*	6.85	*(+62.7)*
Week 78							
Final body weight (g)	38.58	40.62	*(+5.3)*	39.09	*(+1.3)*	31.15**	*(-19.3)*
Absolute liver weight (g)	1.62	1.85	*(+14.2)*	1.91**	*(+17.9)*	2.37**	*(+46.3)*
Relative liver weight (% of body weight)	4.26	4.65	*(+9.2)*	4.90**	*(+15.0)*	7.62**	*(+78.6)*
Females							
Week 52							
Final body weight (g)	36.23	34.14	*(-5.8)*	34.03	*(-6.1)*	26.58**	*(-26.6)*
Absolute liver weight (g)	1.51	1.44	*(-4.6)*	1.54	*(+2.0)*	2.26**	*(+49.7)*
Relative liver weight (% of body weight)	4.21	4.20	*(+0.2)*	4.61	*(+9.5)*	8.39**	*(+99.3)*
Week 78							
Final body weight (g)	32.61	33.77	*(+3.6)*	33.73	*(+3.4)*	27.17**	*(-16.7)*
Absolute liver weight (g)	1.66	1.64	*(-1.2)*	2.20**	*(+32.5)*	2.59**	*(+56.0)*
Relative liver weight (% of body weight)	5.18	4.94	*(-4.6)*	6.62	*(+27.8)*	9.37**	*(+80.9)*

* p ≤ 0.05, ** p ≤ 0.01, statistically different to control

The higher absolute and relative liver weights noted in the animals given 400 and 3,200 ppm at the end of 52- and 78-week treatment periods, were considered to be treatment-related and correlated with the hepatocellular hypertrophy noted among these animals on all occasions.

At the end of 52 and 78 weeks of treatment, some other differences were noted between treated and control animals in the absolute and relative organ weights. However, as these differences were slight, not dose-related, sometimes without similar trend in both sexes and were without correlative relevant histopathological changes, they were considered to be of no toxicological importance.

6. Histopathology

Non-neoplastic findings (52 weeks of treatment):

Treatment-related microscopic findings were noted in the liver comprising dose-related hepatocellular hypertrophy predominantly centrilobular in males and periportal in females at 400 and 3,200 ppm (see Table 5.5- 44).

Table 5.5- 44: Incidence of animals with hepatocellular hypertrophy

Liver findings	Dose level (ppm)							
	Males				Females			
	0	50	400	3,200	0	50	400	3,200
Number of animals examined	10	10	10	10	10	10	10	10
Hepatocellular hypertrophy	0	0	5	10	0	0	6	9

The incidence, severity and morphological characteristics of the other microscopic findings encountered were similar in both control and treated animals and did not show an indication of a treatment-relationship.

Neoplastic findings (52 weeks of treatment):

Hepatocellular adenoma was found in one female given 400 ppm and in three female mice given 3,200 ppm. The higher incidence of hepatocellular adenoma at 3,200 ppm which attained statistical significance ($p < 0.0336$) was considered to be treatment-related (see Table 5.5- 45).

Table 5.5- 45: Incidence of animals with liver tumors

Liver findings	Dose level (ppm)							
	Males				Females			
	0	50	400	3,200	0	50	400	3,200
Number of animals examined	10	10	10	10	10	10	10	10
Hepatocellular adenomas	0	0	0	0	0	0	1	3

Non-neoplastic findings (78 weeks of treatment):

The principal treatment-related non-neoplastic changes were hepatocellular hypertrophy and the higher incidence of altered cell foci as given in Table 5.5- 46.

Table 5.5- 46: Incidence of hepatocellular hypertrophy and altered cell foci

Liver findings	Dose level (ppm)							
	Males				Females			
	0	**50**	**400**	**3,200**	**0**	**50**	**400**	**3,200**
Number of animals examined	50	50	50	50	50	50	50	50
Hepatocellular hypertrophy	0	0	20	49	0	0	41	46
Altered cell foci:								
acidophilic	0	3	1	8	0	1	2	20
basophilic	1	2	0	0	1	0	1	1
clear	0	1	0	2	0	1	0	1
vacuolated	0	0	2	4	0	1	0	2
mixed	0	2	2	4	0	0	1	1
Total altered cell foci	**1**	**8**	**5**	**18**	**1**	**3**	**4**	**25**
(%)	*(2)*	*(16)*	*(10)*	*(36)*	*(2)*	*(6)*	*(8)*	*(50)*

No statistical analyses were performed.

High incidence of hepatocellular hypertrophy predominantly centrilobular in males and periportal in females were noted in the males and females given 400 or 3,200 ppm. This was associated with markedly high incidence of altered cell foci (especially acidophilic) in the males and females given 3,200 ppm.

The other non-neoplastic changes in the liver (such as mononuclear cell aggregation, coagulative hepatic cell necrosis, leucocytosis) were of similar incidence and severity in both control and treated animals and were regarded as being of no toxicological importance.

No treatment-related microscopic findings were note in other organs and tissues.

All non-neoplastic changes encountered in the other organs and tissues, like kidneys, heart, lungs, brain, haemolymphoreticular system, reproductive system and endocrine organs, were noted with comparatively similar incidence and severity in the control and treated groups and showed no indication of a treatment-relationship.

Neoplastic findings (78 weeks of treatment):

The number of animals with neoplasms, the number of animals with more than one primary neoplasm and the number of animals with benign and malignant tumours (see Table 5.5- 47) were comparatively similar in all groups.

Table 5.5- 47: Incidence of animals with neoplasms

Parameter	Dose level (ppm)							
	Males				Females			
	0	50	400	3,200	0	50	400	3,200
Number of animals examined	50	50	50	50	50	50	50	50
Number of animals with neoplasms	17	14	13	16	21	16	17	26
Number of animals with more than one primary neoplasm	4	1	0	3	6	3	1	3
Number of animals with metastases	2	3	0	0	1	2	3	3
Number of animals with benign neoplasms	8	4	7	13	11	7	8	18
Number of animals with unclassified neoplasms	0	0	0	0	0	0	0	0
Number of animals with malignant neoplasms	12	11	6	5	14	10	10	11

No statistical analyses were performed.

At 3,200 ppm, the incidence of hepatocellular adenomas was statistically significantly higher than controls in both sexes. In addition, the incidental tumour analysis as assessed from the evaluation of neoplastic lesions observed at each 100 day-interval in the animals that died or were killed prematurely showed a decrease in the latency of hepatocellular adenoma appearance in the females given 3,200 ppm, when compared with controls (one hepatocellular adenoma already during day 301-400). The incidence and time of onset of the hepatocellular adenomas in the other treated groups (50 and 400 ppm) were comparatively similar to that of the controls (see Table 5.5- 48). Moreover, no increased incidence of hepatocellular carcinoma were observed in any of the groups after the 78-week treatment period.

The incidence of hepatocellular neoplastic lesions is given in Table 5.5- 48.

Table 5.5- 48: Incidence of animals with liver neoplasms

Liver neoplasms	Dose level (ppm)			
	0	50	400	3,200
Males				
Number of animals examined	50	50	50	50
Hepatocellular adenoma *(%)*	5 *(10)*	0 *(0)*	5 *(10)*	11* *(22)*
Incidence in interim death animals / Time period of onset (days) ##	1 / 401-500 1 / 501-558	-	-	1 / 401-500
Hepatocellular carcinoma *(%)*	3 *(6)*	1 *(2)*	0 *(0)*	2 *(4)*
Incidence in interim death animals / Time period of onset (days) ##	1 / 501-558	1 / 501-558		1 / 401-500
Total *(%)*	8 *(16)*	1 *(2)*	5 *(10)*	13 *(26)*
Females				
Number of animals examined	50	50	50	50
Hepatocellular adenoma *(%)*	1 *(2)*	2 *(4)*	0 *(0)*	16** *(32)*
Incidence in interim death animals / Time period of onset (days) ##	-	-	-	1 / 401-400 1 / 501-558
Hepatocellular carcinoma *(%)*	0 *(0)*	0 *(0)*	2 *(4)*	0 *(0)*
Incidence in interim death animals / Time period of onset (days) ##	-	-	-	-
Total *(%)*	1 *(2)*	2 *(4)*	2 *(4)*	16** *(32)*

* $p \leq 0.05$, statistically different to control

** $p \leq 0.0005$, statistically different to control

\# Historical control data (HCD) range from two long term studies (treatment period: 78 weeks) in C57Bl/6 mice performed between November 1998 and May 2000 (149 males, 150 females; Citoxlab- Safety and Health Research Laboratories)

The remaining number of findings of hepatocellular adenoma and carcinoma were observed after terminal sacrifice at study termination.

The neoplastic lesions found in the haemolymphoreticular system (as assessed from the microscopic examination of the liver and lymphoid organs) were diagnosed as lymphocytic malignant lymphoma, malignant lymphoma follicular centre cell, mixed type (syn. composite lymphoma) and histiocytic sarcoma.

The incidence of haematopoietic neoplasms is given in Table 5.5- 49.

Table 5.5- 49: Incidence of animals with haematopoietic neoplasms

Haematopoietic neoplasms	Dose level (ppm)							
	Males				Females			
	0	50	400	3,200	0	50	400	3,200
Number of animals examined	50	50	50	50	50	50	50	50
Lymphocytic malignant lymphoma	0	0	0	0	1	0	0	0
Malignant lymphoma follicular center cell, mixed type	8	5	5	2	11	6	6	9
Histiocytic sarcoma	1	1	0	0	1	1	0	1
Total *(%)*	9 *(18)*	6 *(12)*	5 *(10)*	2 *(4)*	12 *(24)*	7 *(14)*	6 *(12)*	10 *(20)*

From the data cited above, it can be concluded that there was no treatment-related effect on the incidence and distribution of the haematopoietic neoplastic lesions. In addition, the incidence of these lesions is clearly within the range described for C57BL/6 in the literature (up to 20.5% in males and up to 31.3% in females: Frith C.H. (1983)[20]) and within or close to the range of historical control data (males 14-18% and females up to 18-22%; [redacted] 2005; M-263591-01-1).

The incidence and the morphological type of the few neoplastic lesions noted in the uterus and vagina were similar to those described for the mice of this strain and age and showed no indication of a treatment-relationship. Few cases of histiocytic sarcoma were noted in the uterus. However, unlike the histiocytic sarcoma originated from the liver, spleen or lymph nodes, in the uterine lesion large areas may be lacking giant cells.

Other few neoplastic lesions in other organs and tissues were recognized as those commonly occurring in mice of this strain and age and showed no indication of treatment-relationship.

In summary the administration of fluopicolide to C57BL/6 for 78 weeks induced after 52 weeks hepatocellular hypertrophy among the males and females given 400 or 3,200 ppm and a higher incidence of hepatocellular adenoma in the females given 3,200 ppm.

After 78 weeks hepatocellular hypertrophy was noted among the males and females given 400 or 3,200 ppm and a markedly higher incidence of hepatocellular adenoma in the males and females given 3,200 ppm.

III. Conclusion

The test substance fluopicolide was administered daily for 78 weeks by the oral route (dietary admixture) to C57BL/6 mice at 50, 400 or 3,200 ppm.

The treatment with the test substance caused a severe reduction of the body weight gain and food consumption at 3,200 ppm, thus indicating that the MTD was reached, higher liver weights, enlarged liver, masses and nodules in the liver at 400 and 3200 ppm, hepatocellular hypertrophy at 400 and 3,200 ppm, and high incidence of altered cell foci and hepatocellular adenoma at 3,200 ppm. The mode of action for the increased incidence of liver adenomas was found to be CAR-mediated and thus secondary to liver enzyme induction like that of phenobarbital (see, [redacted] 2006; M-275342-01-1 and chapter CA 5.8.2), which is known to be not relevant for tumour formation in humans.

Consequently, under our experimental conditions, the No Observed Adverse Effect level (NOAEL) was 50 ppm (corresponding to 7.9 mg/kg bw/day for the males and 11.5 mg/kg bw/day for the females) for toxicity and 400 ppm (corresponding to 64.5 mg/kg bw/day for the males and 91.9 mg/kg bw/day for the females) for carcinogenicity.

Assessment and conclusion by applicant:

The study was conducted according to OECD TG 451 and is valid and acceptable to investigate the carcinogenic potential of fluopicolide in mice. An increased incidence of liver tumours was observed, secondary to a phenobarbital like, CAR mediated mode of action (as a consequence of liver enzyme induction). A NOAEL of 50 ppm (equivalent to 7.9 and 11.5 mg/kg bw/d in males and females) was determined from this study.

[20] Frith C.H. (1983) Spontaneous lesions in virgin and retired breeder BALB/C and C57BL/6 mice. Lab. Anim. Sci., 33,273-286.

An evaluation of the liver tumour findings in the oncogenicity study in mice is provided in a position paper:

Data Point:	KCA 5.5/03
Report Author:	[redacted]
Report Year:	2006
Report Title:	AE C638206 (Fluopicolide) - Assessment of hepatocellular proliferation and lack of carcinogenicity potential
Report No:	M-275342-01-1
Document No:	M-275342-01-1
Guideline(s) followed in study:	not applicable
Deviations from current test guideline:	not specified
Previous evaluation:	yes, evaluated and accepted Addendum 1 to the DAR (2007)
GLP/Officially recognised testing facilities:	not applicable
Acceptability/Reliability:	Yes

Executive Summary:

The dietary administration of fluopicolide produced higher incidence of hepatocellular adenoma (HCA) in high dose male and female mice following a 78-week treatment period ([redacted] 2003; M-225595-01-1). Given that these HCA were not observed at lower dose levels in mice, not observed in rats following a 2-year treatment period and taken into account the lack of genotoxicity potential of fluopicolide, the higher incidence of HCA was thus considered to be subsequent to a threshold mechanism with a Phenobarbital-like mechanism of action (hepatocellular hypertrophy and transient cell proliferation) which is a well-known mechanism of action specific to the mouse and of no relevance to humans[21, 22, 23].

In a 28-day explanatory toxicity study ([redacted] 2004; M-229594-01-1), fluopicolide was shown to be a strong inducer of total cytochrome P450 and BROD and PROD associated activities. In addition, fluopicolide produced a marked transient liver cell proliferation on Day 7 which returned to control levels on Day 28. These findings were similar to those observed with phenobarbital showing that fluopicolide is a phenobarbital-like compound. Moreover, the PCNA assessment on liver tissue from animals at 3200 ppm ([redacted] 2006; M-205579-02-1) showed that fluopicolide did not produce hepatocellular proliferation on Day 90. This is completely consistent with the lack of cell proliferation observed on Day 28 with the BrdU assessment. These findings emphasize that the transient liver cell proliferation followed by a return to control levels (steady state) is necessary for the

[21] Anderson, M. et al. (1992): Oncogenes in mouse liver tumours. In Klein-Szanto, A.J.P., Anderson, M.W., Barrett, J.C. and Slaga, T.J. (Eds), Comparative Molecular Carcinogenesis. Wiley-Liss, New York, PP.187-20

[22] Grasso, P. et al. (1991): Evidence for and possible mechanisms of nongenotoxic carcinogenesis in rodent liver. Mutation Res., 248: 271-290

[23] Grasso, P. et al. (1991): Role of persistent, non-genotoxic tissue damage in rodent cancer and relevance to humans. Ann. Rev. Pharmacol. Toxicol., 31: 253-287

development of HCA following a long term exposure period to phenobarbital-like product in mice[24, 25, 26].

This mechanism of action is clearly specific to the mouse and of no relevance to humans.

In conclusion, fluopicolide by producing a marked transient liver cell proliferation in high dose mice would allow the development of HCA following a prolonged exposure period. Therefore, in the opinion of Bayer, the higher incidence of HCA observed in high dose male and female mice following a 78-week treatment period with fluopicolide are of no relevance to humans.

Conclusion:

Fluopicolide is thus devoid of any carcinogenicity potential in humans.

Assessment and conclusion by applicant:
Position paper is valid and acceptable to outline the non-human relevant mechanism of the liver tumours found in mice. The mechanism of action is specific to the mouse and not relevant to humans.

[24] Schulte-Hermann, R. (1974). Induction of liver growth by xenobiotic compounds and other stimuli. CRC Crit. Rev. Toxicol., 3: 97-158

[25] Schulte-Hermann, R. (1979: Adaptive liver growth induced by xenobiotic compounds: its nature and mechanism. Arch.Toxicol., Suppl. 2: 113-124

[26] Hildebrand, B. et al. (1991): Validity of considering that early changes may act as indicators for non-genotoxic carcinogenesis. Mutation Res., 248: 217-237

CA 5.6 Reproductive toxicity

In a **preliminary** study treatment levels for a two-generation study in **rats** were examined. Fluopicolide was administered orally, via the diet, at concentration levels of 0, 50, 200, 750 or 2,500 ppm to groups of 8 males and 8 females, 15 days prior to pairing until termination after weaning of the resulting litters. Selected offspring (constituting an F1 generation) continued to receive the diets from about the time of weaning until termination following attainment of sexual maturation.

This preliminary study showed that dietary concentrations of 2,500 ppm of fluopicolide induced general toxicity observed as bodyweight gain reductions in F0 males during the premating period, and in F0 females during the gestation and lactation period. A transient effect in F0 females was also observed during the gestation period at 750 ppm. F1 offspring body weight gains were also reduced at 2,500 ppm. Reproductive parameters were considered to be unaffected by treatment with fluopicolide up to and including the highest tested dose of 2,500 ppm. Therefore, a dose level of maximum up to 2,500 ppm was regarded as suitable as the high concentration in a main study of reproductive performance.

In the main **2-generation study in rats** the influence of fluopicolide on the fertility and reproductive performance of two successive generations was assessed in male and female rats of the Crl: CD® (SD) IGS BR strain. Fluopicolide was administered continuously in the diet at concentrations of 0, 100, 500 or 2,000 ppm to groups of rats throughout the two generations. The F0 generation, which comprised 28 males and 28 females in each group, received the treated diet for 10 weeks before pairing and throughout mating, gestation, littering and lactation. Offspring survival and growth to weaning were evaluated at which point 24 male and 24 female offspring per group were selected to form the F1 generation.

Body weight gain and food consumption were low for adult animals treated at 2,000 ppm throughout the study, with the exception of the low body weight gain which was not apparent in the females following parturition. Oestrous cycles, mating performance, fertility and fecundity were similar in all groups. Gestation length, parturition process and sperm parameters were unaffected by treatment. The return of females to oestrous cycling following lactation was not influenced by treatment in either generation. Sexual maturation, as assessed by the age and bodyweight at the time of attainment of vaginal opening or balano-preputial separation, was also not affected by treatment with fluopicolide at doses up to and including 2,000 ppm.

Litter parameters at birth of the F1 and F2 progeny and their survival to weaning showed no detrimental effects of treatment. Although initial group mean body weight values were similar in all groups, both male and female offspring at 2,000 ppm displayed a similar pattern of significantly reduced body weight from Day 14 through to weaning, coinciding with the time when the offspring start to eat the diet, suggesting a palatability effect and/or systemic toxicity due to direct consumption of the test diet.

At 2,000 ppm, kidney and liver weights were high for parental males and females in both generations, when compared with the controls and a retrospective histopathological examination showed treatment-related findings in both organs (centrilobular hepatocyte hypertrophy and degenerative and regenerative changes in kidneys) at this dose level. Group mean body weight-relative liver weights were also slightly higher for females treated at 500 ppm, when compared with the controls and centrilobular hepatocyte hypertrophy was also present in in males at 500 ppm from both generations. Since these findings are common in the livers of rodents which have been administered xenobiotics, they are considered to be an adaptive change and not a toxic effect of treatment at this dose level.

In conclusion, a dietary concentration of fluopicolide at 500 ppm should be considered as the No Observed Adverse Effect Level (NOAEL). The minimum mean achieved dosages for the F0 animals at this NOAEL (500 ppm) are 25.5 mg/kg bw/day for the males and 32.9 mg/kg bw/day for the females. The No Observed Effect Level (NOEL) for developing offspring is considered to be 500 ppm, equivalent to a minimum mean achieved dosage of 35.8 mg/kg bw/day for F0 females during gestation and lactation based on the decreased bodyweight gain at 2,000 ppm. The NOEL for reproductive parameters is considered to be 2,000 ppm, equivalent to a mean achieved dosage of at least 103.4 mg/kg bw/day for F0 males and at least 127.3 mg/kg bw/day for F0 females before pairing.

A **rat range finding study** was conducted to select suitable doses of fluopicolide for a subsequent developmental toxicity (teratogenicity) study in Sprague Dawley rats. Groups of 4 mated female Sprague Dawley rats received technical fluopicolide in aqueous methylcellulose (1% w/v) by oral gavage once daily at the dose levels of 500 or 1,000 mg/kg bw from Day 7-20 of pregnancy and were sacrificed on Day 21 of pregnancy.

The key maternal findings in this study included a reduced gain in body weight throughout the period of treatment at 1,000 mg/kg bw/day (-34% compared to 500 mg/kg bw/day). Food consumption showed a marked initial (Days 7-10) decrease at this dose level and a slight reduction at 500 mg/kg bw/day. No compound-related effects were observed at necropsy of the animals. Post-implantation loss was elevated at 1,000 mg/kg bw/day and included one total resorption. Mean foetal weight and crown-rump length were reduced at 1000 and 500 mg/kg bw/day. Therefore, the highest dose selected for the definitive study of developmental toxicity was 700 mg/kg bw/day.

In the **main developmental toxicity study in rats**, groups of 23 mated female Sprague Dawley rats received fluopicolide by oral gavage in 1% methylcellulose once daily at the dose levels of 0, 5, 60 or 700 mg/kg bw/day from Day 7-20 of pregnancy (Day 0: day of mating, Day 1: day of sperm detection). They were sacrificed on Day 21 of pregnancy.

Body weights and weight gains were decreased in the animals from the high dose group, especially at the beginning of the treatment period during gestational Days 7-10, a gestational phase which is considered to be highly susceptible to influences regarding skeletal development. These animals showed also a slight initial decrease in food consumption after beginning of treatment.

Mean foetal body weights, crown-rump lengths and placental weights were slightly but statistically significantly decreased in the high dose group. However, litter size, number of live and dead foetuses as well as sex ratios were unaffected by the administration of the test substance. Incidences of early and late conceptuses undergoing resorption were also not affected by the administration of the test compound up to and including the highest tested dose level of 700 mg/kg bw/day.

Morphological examination of the foetuses revealed one foetus with multiple malformations at the vertebral column and pelvis in the intermediate dose group and one foetus with microphthalmia in the high dose group. These findings are considered to be incidental due to their isolated occurrence.

Foetuses from the high dose group showed increased incidences of minor skeletal defects at the thoracic vertebrae, sternebrae and ribs. However, only a small number of foetuses in single litters was affected and these findings are not considered to have adverse consequences for the foetuses in postnatal life. The observations represent mostly a perturbation of ossification, transient in nature, being resolved as ossification progresses. In addition, a delayed ossification was detected at 700 mg/kg bw/day which indicated together with the decreased foetal weight and length a generally retarded foetal development at this maternally toxic dose level.

In conclusion, oral administration of fluopicolide to the pregnant rat at the dose of 700 mg/kg bw/day caused maternal toxicity as evidenced by decreased body weight gains and slightly decreased food consumption. Mean foetal body weights and crown-rump lengths were also slightly decreased at700 mg/kg bw/day. In addition minor defects at the thoracic vertebrae, sternebrae and ribs as well as delayed ossification were observed more frequently at this dose level and are considered secondary to the above described maternal toxicity. Fluopicolide was not teratogenic in this developmental toxicity study in rats.

Fluopicolide did not cause any maternal toxicity or embryotoxicity at the dose of 60 mg/kg bw/day or below. Therefore, with regard to the present study the No Observed Effect Level (NOEL) is 60 mg/kg bw/day for maternal toxicity and for developmental toxicity.

A **rabbit range finding study** was conducted in rabbits in order to select a suitable high dose level of fluopicolide for a subsequent developmental toxicity study in Himalayan rabbits. Groups of 4 mated female Himalayan rabbits received fluopicolide suspended in 1% (w/v) aqueous methyl cellulose by oral gavage once daily at the dose levels of 25, 50, 100, 250, 500 or 1,000 mg/kg bw/day from Day 6-28 of pregnancy (Day 0: day of mating) and were sacrificed on Day 29 of pregnancy.

All animals from the 100, 250, 500 or 1,000 mg/kg bw/day group were found dead, killed moribund or killed after abortion up to Day 23 of the study. At the dose of 50 mg/kg bw/day one animal aborted on Day 29. No clinical signs of toxicity were observed at 25 mg/kg bw/day. Body weight gains and food consumption were decreased during the treatment period at 50, 100, 250, 500 or 1,000 mg/kg bw/day. Body weight gains were not impaired in the animals from the 25 mg/kg bw/day group.

Necropsy findings in the animals found dead, killed moribund or killed after abortion consisted of beige discoloured heart, liver and kidney. In most animals the stomach showed petechial bleedings and in some was filled with feed mash. No macroscopically visible changes were observed at necropsy of the animals from the 25 and 50 mg/kg bw/day group.

One animal from the 25 and 50 mg/kg bw/day group each was not pregnant. The animal of the 50 mg/kg bw/day group which aborted had six dead foetuses. No abnormalities were observed at caesarean section the remaining animals from these groups. Gravid uterus and foetal weights were normal and embryofoetal development was unaffected.

Based on the results of this study, a dose level in the region of 50 mg/kg bw/day was considered to be a suitable high dose for the main study.

In the **main rabbit developmental toxicity study**, groups of 23 mated female Himalayan rabbits received fluopicolide by oral gavage in 1% methylcellulose once daily at the dose levels of 0, 5, 20 or 60 mg/kg bw/day from Day 6-28 of gestation and were sacrificed on Day 29 of gestation.

Three animals of the high dose group were found dead and 15 animals of this group were killed after premature delivery from Day 22-29 of gestation. These animals showed decreased defecation, reduced hay consumption, hypoactivity, bristling coat, pultaceous faeces, and discoloured urine. One animal from the intermediate dose group was killed after premature delivery on Day 28 of gestation. This animal showed decreased defecation and reduced hay consumption. Since up to 20% abortions are covered by historical control data this isolated single premature delivery is considered incidental and not treatment-related.

Body weight gains and food consumption were markedly decreased in the animals from the high dose group. Gravid uterus weights were slightly lower in the animals from at the same dose level.

At necropsy, tautly filled stomach, red liquid in urinary bladder and uterus as well as yellowish discolouration of the liver were observed in single animals from the high dose group. No compound-related effects were observed in the low and intermediate dose group.

Dead foetuses were present in most premature deliveries. Mean foetal body weights, crown-rump lengths and placental weights were decreased in the animals from the high dose group. Litter size, number of live and dead foetuses as well as sex ratios remained unaffected by the administration of the test compound. Likewise, incidences of early and late conceptuses undergoing resorption were not affected by the administration of the test substance. Morphological examination of the foetuses did not reveal any compound-related effects.

Oral administration of fluopicolide to pregnant rabbits at the dose of 60 mg/kg bw/day caused severe maternal toxicity as evidenced by mortality, high incidence of premature delivery and decreases in body weight gain and food consumption. All these findings are considered secondary to severe maternal toxicity as evidenced by mortality and decreases in body weight gain due to drastically reduced feed consumption at the highest tested dose. No teratogenic effects were observed in the foetuses at any dose level.

Fluopicolide did not cause any maternal toxicity or embryotoxicity at the dose of 20 mg/kg bw/day. Therefore, the NOAEL is 20 mg/kg bw/day for maternal toxicity and for developmental toxicity.

In addition, an **external expert for reproductive toxicology** (Mary Moxon has more than 40 years' experience of conducting and supervising developmental and reproduction studies for regulatory purposes, within the industrial sector and contract research organisations. Mary is also experienced in foetal pathology and a former foetal pathology facility manager) was asked for review of the experimental study results in relation to the criteria for classification. Her conclusion supports Bayer's assessment that on the basis of the results of a two-generation reproduction study in the rat and prenatal developmental toxicity studies in rats and rabbits, fluopicolide should not be classified as a reproductive toxicant based on the ECHA Guidance on the application of the CLP criteria.

However, fluopicolide was discussed at the 53rd meeting of the Committee for Risk assessment, at which it was agreed amongst the RAC members that fluopicolide should be classified for reproductive toxicity, category 2 (H361d), based on the increase in minor skeletal findings (in the presence of maternal toxicity) in the rat developmental toxicity study.

Table 5.6- 1: Summary of reproductive studies

Study	NOAEL	LOAEL	Findings at LOAEL	Reference
Pilot reproductive study in rats (diet) 0, 50, 200, 750 and 2,500 ppm [equivalent to 0, 4/4, 17/18, 65/67 and 197/204 mg/kg bw/day for M/F]	Parental toxicity: 200 ppm [17/18 mg/kg bw/day in M/F] Offspring: 750 ppm [65/67 mg/kg bw/day in M/F] Reproductive effects: 2,500 ppm [197/204 mg/kg bw/day in M/F]	Parental toxicity: 750 ppm [65/67 mg/kg bw/day in M/F] Offspring: 2500 ppm [197/204 mg/kg bw/day in M/F] Reproductive effects: >2,500 ppm [197/204 mg/kg bw/day in M/F]	↓ bodyweight gain and food consumption (F) ↓ bodyweight gain	[redacted] 2002; M-215963-01-1
2-generation study in rats (diet) 0, 100, 500 and 2,000 ppm [equivalent to 0, 5.2/6.4, 25.5/32.9 and 103.4/127.3 mg/kg bw/day for M/F premating]	Parental toxicity: 500 ppm [25.5/32.9 mg/kg bw/day in M/F] Offspring: 500 ppm [35.8 mg/kg bw/day] Reproductive effects: 2,000 ppm [103.4/127.3 mg/kg bw/day]	Parental toxicity: 2,000 ppm [103.4/127.3 mg/kg bw/day] Offspring: 2000 ppm [103.4/127.3 mg/kg bw/day] Reproductive effects: >2,000 ppm [103.4/127.3 mg/kg bw/day]	↓ bodyweight gain and food consumption (M/F) ↑ liver and kidney weights (M/F) ↓ spleen weights (F) ↑ incidence of centrilobular hepatocyte hypertrophy ↑ incidence of degenerative and regenerative changes in kidneys (M/F) ↓ bodyweight gain	[redacted] 2003; M-232532-01-1 [redacted] 2004; M-247289-01-1

Study	NOAEL	LOAEL	Findings at LOAEL	Reference
Developmental toxicity range finding study in rats (gavage) 500 and 1,000 mg/kg bw/day	Maternal toxicity: <500 mg/kg bw/day Developmental toxicity: <500 mg/kg bw/day	Maternal toxicity: 500 mg/kg bw/day Developmental toxicity: 500 mg/kg bw/day	↓ food consumption ↓ mean foetal weight and crown-rump length	[redacted] 2000; M-198488-01-1
Developmental toxicity study in rats (gavage) 0, 5, 60 and 700 mg/kg bw/day	Maternal toxicity: 60 mg/kg bw/day Developmental toxicity: 60 mg/kg bw/day	Maternal toxicity: 700 mg/kg bw/day Developmental toxicity: 700 mg/kg bw/day	↓ bodyweight gain and food consumption ↓ mean foetal weight and crown-rump length ↓ ossification ↑ incidence of minor defects	[redacted] 2004; M-202155-02-1
Developmental toxicity range finding study in rabbits (gavage) 25, 50, 100, 250, 500 and 1,000 mg/kg bw/day	Maternal toxicity: 25 mg/kg bw/day Developmental toxicity: 50 mg/kg bw/day	Maternal toxicity: 50 mg/kg bw/day Developmental toxicity: >50 mg/kg bw/day	↓ bodyweight gain and food consumption ↑ incidence of premature deliveries	[redacted] 2000; M-211192-01-1
Developmental toxicity study in rabbits (gavage) 0, 5, 20 and 60 mg/kg bw/day	Maternal toxicity: 20 mg/kg bw/day Developmental toxicity: 20 mg/kg bw/day	Maternal toxicity: 60 mg/kg bw/day Developmental toxicity: 60 mg/kg bw/day	↑ mortality and ↓ in body weight gain due to drastically reduced feed consumption ↑ incidence of premature deliveries ↓ mean foetal weight and crown-rump length	[redacted] 2004; M-202513-02-1
Supplementary information: External expert statement regarding fluopicolide's reproductive and developmental toxicity potentials	-	-	This document contains a summary and review of the two-generation and the developmental toxicity studies with rats and rabbits by an external expert for reproductive toxicity concluding that on the basis of the available studies, fluopicolide should not be classified as a reproductive toxicant.	[redacted] 2018; M-638869-01-1

M = male F = female

CA 5.6.1 Generational studies

Data Point:	KCA 5.6.1/01
Report Author:	[redacted]
Report Year:	2002
Report Title:	AE C638206 - Preliminary study of effects on reproductive performance in CD rats by dietary administration
Report No:	C023472
Document No:	M-215068-01-1
Guideline(s) followed in study:	not applicable
Deviations from current test guideline:	Method: none; Study: As a dose-range finding study the study was not intended to comply with OECD guidelines.
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

This preliminary study was performed to enable selection of treatment levels for a two-generation study.

For this purpose, fluopicolide was administered orally, via the diet, at inclusion levels of 0, 50, 200, 750 or 2,500 ppm to groups of 8 males and 8 females. Treatment started 15 days prior to pairing and continued uninterrupted until termination after weaning of the resulting litters. Selected offspring (constituting an F1 generation) continued to receive the diets from about the time of weaning until termination following attainment of sexual maturation. Exposure to fluopicolide was achieved for the F0 adults and the F1 generation throughout the study.

The general condition of the F0 animals remained similar in all groups. One female receiving 2,500 ppm was killed due to parturition problems (dystocia) on Day 22 of gestation. The isolated nature of this finding suggested that it was more likely to be coincidental than treatment-related.

Body weight gains were lower (-22%) for males treated at 2,500 ppm at the end of the premating period. For females treated at 2,500 ppm, body weight gains were also lower before pairing and during gestation days 0-13 (approx. -20%). A transient effect in F0 females was also observed during the gestation period at 750 ppm. Food consumption was reduced for females treated with 2,500 ppm for most periods during gestation, for gestation periods days 3-5 and 6-9 also at 750 ppm, and during lactation at 2,500 ppm (Days 7-13). The achieved intake during the first few days of treatment was slightly lower for the F0 animals treated at 2,500 ppm than expected suggesting a transient palatability effect. The mean compound intakes during the pre-mating period were approx. 4, 17, 65 and 197 mg/kg bw/day for males and 4, 18, 67 and 204 mg/kg bw/day for females at 50, 200, 750 and 2,500 ppm, respectively.

Oestrous cycles, mating performance, fertility and fecundity were considered to be unaffected by treatment with fluopicolide. Gestation length appeared to be slightly longer for the females treated at 2,500 ppm than the controls, but the group size was too low to determine whether this was a true treatment-related finding. Gestation index and the parturition process were considered to be unaffected. Litter parameters incorporating litter size, offspring survival and sex ratio were considered not to be affected by treatment with fluopicolide.

The general condition of the F1 generation was similar in all groups and there were no deaths. Body weight gains for the F1 offspring in the group treated at 2,500 ppm were 87% and 85% of control groups for males and females respectively from approx. Day 14 of age, coinciding with the time when the offspring start to eat the diet, suggesting a palatability effect and/or systemic toxicity due to direct consumption of the test diet. Food consumption and body weight gains continued to be lower for animals

treated at 2,500 ppm following the pattern established before weaning. The timing of balano-preputial separation for the F1 animals was unaffected by treatment. There appeared to be a slight delay in vaginal opening for females treated at 2,500 ppm when compared to the concurrent control. However, a treatment-relation is questionable.

No treatment-related findings were seen at macroscopic examination of the F0 or any of the F1 animals.

This preliminary study showed that dietary concentrations of 2,500 ppm of fluopicolide induced toxicity observed as body weight gain reductions in F0 in both sexes during the premating period, and in F0 females during the gestation period. A transient effect in F0 females was also observed during the gestation period at 750 ppm. F1 offspring bodyweight gains were also reduced at 2,500 ppm from approx. Day 14 of age. Therefore, a dose level up to 2,500 ppm would be suitable as the high concentration in a main study of reproductive performance. This concentration would be expected to elicit a measurable degree of parental toxicity without affecting fertility or reproductive performance.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 (fluopicolide)
Purity:	95.9%
Batch no.:	OP2050046

2. Vehicle and/or positive control

Vehicle:	Basal diet, no positive control

3. Test animals

Species:	Rat
Strain:	Crl:CD® (SD)IGS BR strain (Sprague-Dawley origin)
Age:	Males: 9-10 weeks, females: 8-9 weeks
Weight at start:	Males: 280 to 317 g, females: 191 to 219 g.
Source:	[redacted]
Acclimation phase:	Yes
Diet:	Powdered laboratory animal diet (UAR VFR1 Certified, manufactured by Usine d'Alimentation Rationnelle in France) supplied by Charles River UK, Margate, Kent, England
Water:	Polythene or polycarbonate bottles with sipper tubes
Housing:	The animal room had its own supply of filtered air, which was passed to the atmosphere without recirculation. Housing in TR18 cages from Arrowmight Biosciences, Hereford, England or RB3 modified and RB3 cages from North Kent Plastic Cages Limited, Erith, Kent, England. The cages consisted of stainless steel (TR18) or high-density polypropylene (RB3 and RB3 modified) bodies with lids of stainless steel grid. TR18 and RB3 modified cages had stainless steel grid floors.
Temperature:	19-23 °C
Humidity:	40-70%
Air changes:	Not given (see above)
Photoperiod:	12 hours

B. Study design

1. In life dates: February 21 to May 25, 2001

2. Animal assignment and treatment:

On receipt, animals were non-selectively allocated to cages, up to four of one sex being placed in each cage. Before commencement of treatment, the animals within each sex were ranked by body weight into blocks (5 g range). Surplus animals were discarded from amongst those with outlying body weights and 40 animals of each sex were allocated to the five groups, and re-housed four of one sex/cage, by selecting animals from each body weight range in rotation. The groups contained populations of rats with comparable initial mean body weight and body weight range.

Individuals were then assigned a number and identified by tail tattoo.

The study design is given in Table 5.6.1- 1.

Table 5.6.1- 1: Study design – F0 generation

Test group	Dose level (ppm)	Number of animals	
		Male	Female
1	0	8	8
2	50	8	8
3	200	8	8
4	750	8	8
5	2,500	8	8

Cage labels, identifying the occupants by experiment, animal number, sex and treatment group, were colour-coded.

3. Diet preparation and analysis:

Formulations were prepared on a weekly basis. A pre-mix, of a suitable dietary concentration, was prepared by adding an approximately equal quantity of plain diet to the required weight of fluopicolide and mixing using a spoon. A further amount of plain diet that approximately equalled this mixture was then added and stirred with a spoon. This doubling-up procedure was followed until a visibly homogenous premix of the required weight was achieved, and the premix blended in a Turbula Mixer. A second premix was formulated from this first premix using the doubling-up procedure described above and finally blended in a Turbula Mixer.

The 2,500 and 750 ppm formulations were prepared by direct dilution of the first pre-mix with further quantities of plain diet, and the 200 and 50 ppm formulations were prepared by direct dilution of the second premix with plain diet. Blending was achieved by mixing in a Turbula Mixer.

Dosages and concentrations were expressed in terms of the test material as supplied. F0 males and females were fed the diets for 15 days before pairing for mating. Treatment was continued throughout mating and until termination after weaning of the litters. Animals selected to form the F1 generation received the diets from the time of weaning until termination following sexual maturation.

Information on the homogeneity of mixing, stability and concentration of the test substance in the diet were determined by Huntingdon Life Sciences. The homogeneity and the stability, during ambient temperature storage for 22 days, were confirmed for fluopicolide in VRF 1 formulation at nominal concentrations of 50 and 2,500 ppm. The storage period represented the maximum time from preparation to completion of use.

Additionally, samples of the formulations were taken for the first and last study preparations and analysed by Huntingdon Life Sciences for test substance content. The mean concentrations of fluopicolide in test diet formulations analysed during the study were between 90.8% and 101% of nominal concentrations and were considered satisfactory.

4. Statistics

Statistical evaluation of data was only performed where considered appropriate. The following parameters were analysed, and results are presented in relevant tables of this report:

Bodyweights and bodyweight change, food consumption of females during gestation and lactation, and litter data including offspring body weights.

For data recorded and/or processed by the Xybion computer system (adult organ weights and weekly body weight change) for the parental animals, homogeneity of variance was assessed using Bartlett's test (Bartlett, 1937). Whenever this was found to be statistically significant a Behrens-Fisher test (Cochran and Cox, 1957) was used to perform pairwise comparison, otherwise a Dunnett's test (Dunnett, 1955/64) was used.

For the remaining data statistical analysis was performed using an in-house programme developed by Huntingdon Life Sciences which used the following criteria:

If 75% of the data (across all groups) shared the same value, then a frequency analysis was applied.

Treatment groups were compared using a Mantel test for a trend in proportions (Mantel 1963) and also pairwise Fisher's Exact tests (Fisher 1973) for each dose group against the control.

If Bartlett's test for variance homogeneity (Bartlett 1937) was not significant at the 1% level, or if it was not significant after first a logarithmic or second a square-root transformation, then parametric analysis was applied. If the F1 test for monotonicity of dose-response (Healey 1999) was not significant at the 1% level, then Williams' test was performed for a monotonic trend (Williams 1971, 1972). If the F1 test was significant, showing that the dose-response was not monotonic, then Dunnett's test (Dunnett 1955, 1964) was applied instead. Both the Williams' and Dunnett's test use the error mean square from a one-way analysis of variance.

If Bartlett's test was significant at the 1% level, even after transformation, then non-parametric analysis was applied to the untransformed data. If the H1 test for monotonicity of dose-response (Healey 1999) was not significant at the 1% level, then Shirley's test was applied for a monotonic trend (Shirley 1977). If the H1 test was significant, showing that the dose-response was not monotonic, then Dunn's test (Dunn 1964) was applied instead.

Significant (i.e. $p<0.05$) inter-group differences from the control are reported.

C. Methods

1. Observations

All animals were inspected regarding clinical signs at least twice daily throughout the study and any visible signs of reaction to treatment were recorded, with details of type, severity, time of onset and duration.

Animals killed for reasons animal welfare were subjected to a thorough macroscopic examination of the visceral organs and specimens of abnormal tissues were retained.

2. Body weight and food intake

Males were weighed on the first day of treatment and twice weekly until termination. Females were weighed on the first day of treatment and then twice weekly until mating was detected. Subsequently the females were weighed on Days 0, 6, 13 and 20 after mating and on Days 1, 4, 7, 14 and 21 of lactation. F1 selected animals were weighed weekly from nominal 4 weeks of age until termination following sexual maturation (approx. 8 weeks of age).

Food consumption was recorded twice weekly for the F0 animals until they were paired for mating.

Food consumption for the females was recorded Days 0-2, 3-5, 6-9, 10-12, 13-16 and 17-19 after mating and Days 1-3, 4-6 and 7-13 during lactation.

Food consumption for the F0 selected animals was recorded weekly for nominal Weeks 4 to 5 and 5 to 6 of age - described as Weeks 4 or 5 in the results.

3. Oestrous cycles

For 10 days before pairing of the F0 animals, vaginal smears were taken daily from all females to establish the normality or otherwise of the oestrous cycle. This was continued after pairing with the male until evidence of mating was observed.

4. Mating

After the scheduled period of treatment, the F0 females were paired on a one-to-one basis with males from the same treatment group.

Each morning following pairing, the trays beneath the cages were checked for ejected copulation plugs and a vaginal smear was prepared from each female and examined for the presence of spermatozoa. The day on which a sperm positive vaginal smear or at least three copulation plugs were found was designated Day 0 of gestation. Once mating had occurred, the males and females were separated, and vaginal smearing discontinued.

The pre-coital interval (females only), i.e. the time elapsing between initial pairing and detection of mating was recorded.

5. Parturition and duration of gestation

From Day 20 after mating, the females were inspected three times daily for the onset, progress and completion of parturition.

All females were permitted to deliver their young naturally and rear their own offspring until Day 21 of lactation (litter standardisation was performed at Day 4 of lactation).

6. Observations of litters

All offspring were examined at approx. 24 hours after birth (Day 1), and each live offspring was identified within the litter by a toe tattoo. The following was recorded for each litter:

a) Number born (live and dead).
b) Individual bodyweights of live offspring.
c) Individual sexes.
d) Observations on individual offspring.

Clinical signs, mortality:

Litters were observed daily on clinical signs for evidence of ill health or a reaction to treatment.

Mortality and litter size was checked, daily records were maintained of mortality and consequent changes in litter size. Wherever possible, any offspring found dead were examined externally and internally.

On Day 4 of age, litters containing more than ten offspring were reduced to ten by random culling, leaving, wherever possible, five male and five female offspring in each litter.

Sex ratio:

The offspring were sexed on Days 1, 4 (before and after culling) and 21 of age.

Body weight:

The offspring were weighed individually on Days 1, 4 (before culling), 7, 14 and 21 of age.

F1 selection:

Following weaning, 12 male and 12 female offspring from the F1 litters in each of the groups were selected to form the F1 generation.

Where possible, at least one male and one female were selected from each litter using random numbers within litters after grossly atypical animals had been excluded. Additional male/female offspring were then selected from litters chosen at random within each group to achieve twelve male and twelve female offspring per group. The animals were assigned a number and identified by tail tattoo.

Table 5.6.1- 2: Study design – F1 generation

Test group	Dose level (ppm)	Number of animals	
		Males	Females
1	0	12	12
2	50	12	12
3	200	12	12
4	750	12	12
5	2,500	12	12

The design conditions and serial observations were as described for the first generation.

Sexual maturation:

Sexual maturation of the selected animals was assessed as follows:

Males: Examined daily from Day 38 of age until balano-preputial separation occurred. Body weight was recorded on the day of completion.

Females: Examined daily from Day 28 of age until vaginal opening occurred. Body weight was recorded on the day of vaginal opening.

7. Necropsy, pathology:

All adult animals, weaned animals and any late neonates (> 10 days of age) that were killed for reasons of animal welfare were killed by carbon dioxide inhalation.

Offspring culled on Day 4 of age, and any that were killed for reasons of animal welfare before Day 10 of age, were killed by an intraperitoneal injection of sodium pentobarbitone.

Any parental animals or offspring that were found dead or killed for reasons of animal welfare were subjected to external and internal necropsy examination as soon as possible. Those found dead outside the normal working day were stored in a refrigerator, designated for this purpose, and necropsied the following day.

F0 males were killed after the females had successfully littered. Females that littered and reared offspring to weaning were killed after their respective litters were weaned.

Macroscopic pathology F0 animals:

All animals were subjected to a detailed macroscopic examination for evidence of disease or adverse reaction to treatment. Samples of abnormal tissues were weighed and retained in appropriate fixative. For all females, the number of implantation sites was recorded.

Offspring (sporadic deaths and unselected F1 offspring):

For early neonates that were found dead, an assessment of the stomach for milk content was made.

Offspring culled on Day 4 of age that were grossly normal were discarded without macroscopic examination.

Any deaths in late neonates or weaned offspring not selected for continuation of the study (killed at approx. 4 weeks of age) were examined externally and internally for macroscopic abnormalities. Specimens of abnormal tissues were retained in industrial methylated spirit.

F1 selected animals:

Males and females were killed after sexual maturation was complete (at approx. 7 to 8 weeks of age) and were subjected to gross necropsy. The animals were examined externally and internally for macroscopic abnormalities. Specimens of abnormal tissues were retained in industrial methylated spirit pending possible future examination.

II. Results and Discussion

A. Observations

F0 generation

1. Clinical signs, mortality - F0 generation

Dosages of up to 2,500 ppm, administered by the dietary route, had no effect upon the general condition of the animals and there were no signs that were considered to be treatment-related.

One female (Animal Number 1078) receiving 2,500 ppm was killed due to parturition problems (dystocia) on day 22 of gestation. Findings at necropsy included red staining around the uro-genital region and lower ventral abdomen, reduced and dehydrated contents in the caecum, few and very firm faecal pellets in the colon and rectum and a small pale spleen. The uterus contained twenty implantations, one empty site and one site containing only a placenta. The isolated nature of the parturition problems observed for this animal suggested that it was more likely to be coincidental than treatment-related. Apart from this female there were no other mortalities among adult animals.

2. Body weight and food intake - F0 generation

Body weight:

In males, body weight gains were lower at 2,500 ppm compared to controls; the divergence was apparent from the first week of treatment (Days 0-7: -25%, $p < 0.01$) and persisted for the 7 weeks of treatment (Days 0-49: -22%, $p < 0.01$). Body weight gains in the other male groups were considered to be largely unaffected with no clear dosage related pattern emerging.

In females, bodyweight gains were slightly lower before pairing (Days 0-14: -20%, not statistically significant) and during gestation (approx. 82-92% of Control values, $p < 0.05$) for females at 2,500 ppm when compared with the controls. A transient effect in F0 females was also observed during the gestation period at 750 ppm. No clear differences were apparent during lactation.

An overview is given in Table 5.6.1-3.

Table 5.6.1-3: Body weight and body weight gains (g) of parental animals (F0 generation)

	Generation	Dose level [ppm] 0	50		200		750		2,500	
Males										
Body weights [g] (*% difference to control*)										
Day 0	**Pre- and postmating**	301	302	*(±0)*	302	*(±0)*	294	*(-2)*	292	*(-3)*
Day 7		361	354	*(-2)*	361	*(±0)*	351	*(-3)*	337	*(-7)*
Day 14		420	404	*(-4)*	408	*(-3)*	399	*(-5)*	379	*(-10)*
Day 21		455	437	*(-4)*	443	*(-3)*	427	*(-6)*	411	*(-10)*
Day 28		485	468	*(-3)*	471	*(-3)*	453	*(-7)*	433	*(-11)*
Day 35		509	497	*(-2)*	504	*(-1)*	488	*(-4)*	458	*(-10)*
Day 42		546	534	*(-2)*	530	*(-3)*	519	*(-5)*	489	*(-10)*
Day 49		572	560	*(-2)*	550	*(-4)*	540	*(-6)*	504	*(-12)*

	Generation	Dose level [ppm] 0	50		200		750		2,500	
Body weight gains [g] (*% difference to control*)										
Day 0-7		60	52	*(-13)*	59	*(-2)*	57	*(-5)*	45**	*(-25)*
Day 0-14		119	102	*(-14)*	106	*(-11)*	105	*(-12)*	87**	*(-27)*
Day 0-49		271	258	*(-5)*	248	*(-9)*	246	*(-9)*	212**	*(-22)*
Females										
Body weights [g] (*% difference to control*)										
Day 0	**Premating**	205	203	*(-1)*	205	*(±0)*	204	*(±0)*	208	*(+2)*
Day 7		230	228	*(-1)*	231	*(±0)*	223	*(-3)*	223	*(-3)*
Day 14		246	245	*(±0)*	251	*(+2)*	243	*(-1)*	240	*(-2)*
Body weight gains [g] (*% difference to control*)										
Day 0-7		25	25	*(±0)*	25	*(±0)*	18	*(-28)*	15	*(-40)*
Day 0-14		40	42	*(+5)*	46	*(+15)*	39	*(-2)*	32	*(-20)*
Body weights [g] (*% difference to control*)										
GD 0	**Gestation**	255	257	*(+1)*	258	*(+1)*	254	*(±0)*	249	*(-2)*
GD 6		292	296	*(+1)*	300	*(+3)*	285	*(-2)*	280	*(-4)*
GD 13		334	335	*(±0)*	342	*(+2)*	327	*(-2)*	313	*(-6)*
GD 20		418	426	*(+2)*	436	*(+4)*	415	*(-1)*	399	*(-4)*
Body weight gains [g] (*% difference to control*)										
GD 0-6		38	40	*(+5)*	43	*(+13)*	31	*(-18)*	31	*(-18)*
GD 0-13		80	79	*(-1)*	85	*(+6)*	73	*(-9)*	65*	*(-19)*
GD 0-20		163	169	*(+4)*	178	*(+9)*	161	*(-1)*	150	*(-8)*
Body weights [g] (*% difference to control*)										
LD 1	**Lactation**	313	321	*(+3)*	330	*(+5)*	310	*(-1)*	291	*(-7)*
LD 4		328	331	*(+1)*	348	*(+6)*	329	*(±0)*	314	*(-4)*
LD 7		347	345	*(-1)*	363	*(+5)*	336	*(-3)*	325	*(-6)*
LD 14		360	367	*(+2)*	370	*(+3)*	358	*(-1)*	337	*(-6)*
LD 21		350	360	*(+3)*	363	*(+4)*	349	*(±0)*	334	*(-5)*
Body weight gains [g] (*% difference to control*)										
LD 1-4		15	11	*(-27)*	18	*(+20)*	19	*(+27)*	22	*(+47)*
LD 1-7		34	24	*(-29)*	33	*(-3)*	26	*(-23)*	34	*(±0)*
LD 1-14		47	46	*(-2)*	40	*(-15)*	48	*(+2)*	46	*(-2)*
LD 1-21		38	39	*(+3)*	33	*(-13)*	39	*(+3)*	43	*(+13)*

GD: gestation day; LD: lactation day
* / ** statistically significant difference from control, $p \leq 0.05$ / $p \leq 0.01$

Food intake:

In males, food consumption recorded during the two-week pre-pairing phase, was slightly low (approx. 74-90% of control values) for males receiving 2,500 ppm when compared with the controls.

In females, during the first week of treatment, food consumption was slightly low (approx. 75-90% of control value) for females receiving 2,500 ppm when compared with the controls. This trend did not continue into the second week but re-appeared during gestation (approx. 82-93% of control values, $p < 0.05$ for 4/6 periods). Food consumption was also low (approx. 90-93% of control values, $p < 0.05$ for 2/6 periods) during gestation for females receiving 750 ppm. No clear effects of treatment were apparent for food consumption during lactation apart from a possible reduction at 2,500 ppm which developed during the second week (approx. 83% of control values, $p < 0.01$).

The other dose groups were considered to be unaffected. The following tables give an overview.

The only consistent effect on food conversion efficiency was that it appeared slightly low for both sexes receiving 2,500 ppm when compared with the controls which was statistically not significant.

Table 5.6.1- 4: Food consumption (g/rat/week) prior to pairing – F0 generation

Time period	Dose level (ppm)									
	Males					Females				
	0	50	200	750	2,500	0	50	200	750	2,500
Day 1-14	106	120	118	119	95	81	79	83	78	74
(% control)		*(113)*	*(111)*	*(112)*	*(90)*		*(97)*	*(102)*	*(96)*	*(91)*

Table 5.6.1- 5: Food consumption (g/rat/day) of dams during gestation (g) - F0 generation

Time period	Dose level (ppm)				
	0	50	200	750	2,500
Days 0-2	27	27	28	25	24
Days 3-5	28	29	29	26*	26**
(% control)				(93)	(93)
Days 6-9	30	29	30	27**	26**
(% control)				(90)	(87)
Days 10-12	29	29	30	27**	27**
(% control)				(93)	(93)
Days 13-16	29	28	31	27	27
Days 17-19	33	31	33	30	27*
(% control)					(82)

* $p \leq 0.05$; ** $p \leq 0.01$ statistically significantly different to controls
(% of controls if statistically significant difference)

Table 5.6.1- 6: Food consumption (g/rat/day) of dams during lactation – F0 generation

Time period	Dose level (ppm)				
	0	50	200	750	2,500
Days 1-3	42	40	43	39	45
Days 4-6	54	52	52	52	49
Days 7-13	75	73	77	70	62**

** $p \leq 0.01$ statistically significantly different to controls

Achieved dosages:

The achieved dosage at all dietary concentrations for both sexes was considered to be satisfactory and sufficient exposure to fluopicolide was achieved. During the first few days of treatment the achieved intake at 2,500 ppm was slightly lower than might have been expected (counter to the usual pattern of higher values initially, declining as the animals grow). This could suggest a transient palatability effect. Fluctuations during gestation and lactation were in line with expectation and considered to be related to changes in the physiological demands during these periods that affected the homeostasis of the females. The mean compound intakes during the pre-mating period were approx. 4, 17, 65 and 197 mg/kg bw/day for males and 4, 18, 67 and 204 mg/kg bw/day for females at 50, 200, 750 and 2,500 ppm, respectively.

Table 5.6.1- 7: Achieved dosage (mg/kg bw/day) before pairing – F0 generation

Time period	Dose level (ppm)									
	Males					Females				
	0	50	200	750	2,500	0	50	200	750	2,500
Days 1-3	-	5	18	68	194	-	5	18	65	178
Days 4-7	-	4	17	64	208	-	4	18	67	209
Days 8-10	-	4	16	67	201	-	4	18	70	225
Days 11-14	-	4	15	59	185	-	4	18	64	208

Table 5.6.1- 8: Achieved dosages (mg/kg bw/day) during gestation – F0 generation

Time period	Dose level (ppm)				
	0	50	200	750	2,500
Days 0-6	-	5	21	73	236
Days 6-13	-	5	19	67	220
Days 13-20	-	4	16	58	192

Table 5.6.1- 9: Achieved dosages (mg/kg bw/day) during lactation – F0 generation

Time period	Dose level (ppm)				
	0	50	200	750	2,500
Days 1-4	-	6	26	92	370
Days 4-7	-	8	29	116	384
Days 7-14	-	10	42	151	471

3. Reproductive results – F0 generation

Oestrous cycles:

The regularity and duration of the oestrous cycles were unaffected at dosages of up to 2,500 ppm.

Mating performance and fertility:

Mating performance and fertility were unaffected by treatment as can be seen in Table 5.6.1- 10.

Table 5.6.1- 10: Mating performance and fertility – F0 generation

Parameter	Dose level (ppm)				
	0	50	200	750	2,500
Number mating	8	8	8	8	8
Number achieving pregnancy	8	8	8	8	8
Conception rate (%)	100	100	100	100	100

Gestation length, gestation index and parturition:

Gestation length was within the usual range of 22 to 23.5 days for all females. The gestation lengths for females receiving 2,500 ppm appeared to be slightly longer than the controls, but all remained within 23 days. This slight shift was possibly attributable to the slightly lower body weights of these females but the group size was too low to be certain whether this was a true treatment related finding.

Gestation index was slightly low for animals in group 5 (2,500 ppm), but this was attributable to the one female with parturition problems on Day 22 of gestation and not related to treatment with fluopicolide.

Table 5.6.1- 11: Gestation length and gestation index – F0 generation

Parameter	Dose level (ppm)				
	0	50	200	750	2,500
Number of pregnant animals	8	8	8	8	8
Number of litters born	8	8	8	8	7
Gestation index (%)	100	100	100	100	88
No. of animals with defined gestation length (% of animals)					
Gestation length: 22 days	5 (63)	5 (63)	4 (50)	5 (63)	1 (13)
Gestation length: 22.5 days	1 (13)	2 (25)	4 (50)	2 (25)	3 (38)
Gestation length: 23 days	2 (25)	1 (13)	0 (0)	1 (13)	4 (50)

Offspring results:

Litter responses

The general condition of the offspring was unaffected at any dosage.

Litter size and offspring survival were not affected. The numbers of implantation sites, total litter size and live litter size on day 1 of age and offspring survival assessed by post-implantation survival, live birth, viability and lactation indices were considered to be similar in all groups.

Regarding sex ratio, the ratio of male to female offspring showed some inter-group variation in particular with a slightly lower proportion of males to females at 2,500 ppm. This difference was considered incidental and no effect of treatment on sex ratio was indicated.

An overview is given in Table 5.6.1- 12.

Table 5.6.1- 12: Uterine implantations, litter data

Parameter	Dose level (ppm)				
	0	50	200	750	2,500
Litter number	8	8	8	8	8
Implantations (mean)	15.5	16.6	16.0	17.1	15.7
Total litter size on day 1	14.6	15.8	15.0	16.1	14.6
Post implantation survival index (%)	94.3	94.6	94.0	93.7	92.5
Live birth index (%)	100.0	100.0	99.1	98.5	100.0
Viability index (%)	99.3	98.3	99.3	99.4	97.4
Lactation index (%) – day 7	98.8	100.0	100.0	100.0	92.9
Lactation index (%) – day 21	98.8	100.0	100.0	100.0	92.9
Sex ratio (day 1):					
Males	7.5	8.6	7.6	8.4	6.6
Females	7.1	7.1	7.4	7.8	8.0
Sex ratio (day 21):					
Males	4.8	5.0	5.4	5.3	4.1
Females	5.1	5.0	4.6	4.8	5.1

Offspring body weights (F1 pups)

Overall group mean body weight gains from Day 1 to 21 of age were slightly low for both male (approx. 87% of control values, $p < 0.05$) and female (approx. 85% of control values, $p < 0.05$) offspring in Group 5 (2,500 ppm) when compared with the controls. These lower body weights first appeared on Day 14 of age, coinciding with the time when the offspring started to eat the diet suggesting a palatability effect and/or systemic toxicity due to direct consumption of the test diet and not a lactational effect. Body weight gains in the other groups were considered to be unaffected.

An overview is given in Table 5.6.1- 13.

Table 5.6.1- 13: Body weight and body weight gains (g) of F1 pups

	Dose level (ppm)									
	0	50		200		750		2,500		
Males										
Body weights [g] (*% difference to control*)										
Day 1 (before cull)	6.8	6.8	*(±0.0)*	6.7	*(-1.5)*	6.8	*(±0.0)*	7.1	*(+4.4)*	
Day 4 (before cull)	9.5	9.4	*(-1.1)*	9.2	*(-3.2)*	8.9	*(-6.3)*	9.3	*(-2.1)*	
Day 4 (after cull)	9.6	9.6	*(±0.0)*	9.2	*(-4.2)*	8.9	*(-7.3)*	9.7	*(+1.0)*	
Day 7 (after cull)	14.7	15.3	*(+4.1)*	14.8	*(+0.7)*	14.2	*(-3.4)*	15.3	*(+4.1)*	
Day 14 (after cull)	32.2	32.1	*(-0.3)*	31.9	*(-0.9)*	30.4	*(-5.6)*	29.9	*(-7.1)*	
Day 21 (after cull)	51.5	49.8	*(-3.3)*	50.5	*(-1.9)*	47.6	*(-7.6)*	46.0	*(-10.7)*	
Body weight gain (g) Day 1-21	44.7	43.0	*(-3.8)*	43.8	*(-2.0)*	40.8	*(-8.7)*	38.8*	*(-13.2)*	
Females										
Body weights [g] (*% difference to control*)										
Day 1 (before cull)	6.3	6.4	*(+1.6)*	6.4	*(+1.6)*	6.5	*(+3.2)*	6.7	*(+6.3)*	
Day 4 (before cull)	9.0	8.8	*(-2.2)*	8.8	*(-2.2)*	8.5	*(-5.6)*	8.9	*(-1.1)*	
Day 4 (after cull)	9.0	8.9	*(-1.1)*	8.9	*(-1.1)*	8.7	*(-3.3)*	9.0	*(±0.0)*	
Day 7 (after cull)	14.1	14.4	*(+2.1)*	14.4	*(+2.1)*	14.1	*(±0.0)*	13.7	*(-2.8)*	
Day 14 (after cull)	31.4	30.2	*(-3.8)*	31.1	*(-1.0)*	30.1	*(-4.1)*	25.1*	*(-20.1)*	
Day 21 (after cull)	48.8	46.3	*(-5.1)*	48.5	*(-0.6)*	46.3	*(-5.1)*	42.9*	*(-12.1)*	
Body weight gain (g) Day 1-21	42.5	39.9	*(-6.1)*	42.1	*(-0.9)*	39.9	*(-6.1)*	36.2*	*(-14.8)*	

* $p \leq 0.05$ statistically significantly different to controls

Examination results of unselected offspring and F0 parents

Necropsy of unselected offspring and F0 adults

There were no macroscopic observations at necropsy that suggested any test article-related effects of treatment upon the offspring.

Macroscopic examination of the F0 animals at necropsy revealed no test article-related findings that were considered to be related to the dietary administration of fluopicolide.

F1 generation

1. Clinical signs, mortality - F1 generation

Dosages of up to 2,500 ppm, administered by the dietary route, had no effect upon the general condition of the animals and there were no signs that were considered to be treatment-related. There were no deaths in the F1 generation.

2. Body weight and food intake - F1 generation

Body weight gain was statistically significantly decreased in males at 2,500 ppm (Week 1-3: -11% compared to control) and also slightly reduced in females.

Body weight gains in the other groups were considered to be unaffected.

Table 5.6.1- 14: Body weight and body weight gains (g) of F1 animals

Timepoint #	Dose level (ppm) 0	50		200		750		2,500	
Males									
Body weights [g] (*% difference to control*)									
Week 1	148	147	*(+0.7)*	156	*(+5.4)*	148	*(±0.0)*	131	*(-11.5)*
Week 2	210	210	*(±0.0)*	218	*(+3.8)*	207	*(-1.4)*	188	*(-10.5)*
Week 3	277	277	*(±0.0)*	283	*(+2.2)*	271	*(-2.2)*	244	*(-11.9)*
Body weight change (g) Week 1-3	186	187	*(+0.5)*	186	*(±0.0)*	180	*(-3.2)*	165*	*(-11.3)*
Females									
Body weights [g] (*% difference to control*)									
Week 1	126	124	*(-1.6)*	136	*(+7.9)*	128	*(+1.6)*	116	*(-7.9)*
Week 2	161	160	*(-0.6)*	171	*(+6.2)*	165	*(+2.5)*	151	*(-6.2)*
Week 3	190	186	*(-2.1)*	200	*(+5.3)*	196	*(+3.2)*	178	*(-6.3)*
Body weight change (g) Week 1-3	106	106	*(±0.0)*	110	*(+3.8)*	113	*(+6.6)*	103	*(-2.8)*

* $p \leq 0.05$ statistically significantly different to controls
\# F1 selected animals were weighed weekly from nominal 4 weeks of age until termination following sexual maturation (approximately 8 weeks of age).

Food consumption was slightly low (not statistically significant) for animals receiving 2,500 ppm when compared with the controls (approx. 87-90% of control values in males and 91-93% in females). The other groups were unaffected.

Overall food conversion efficiency for the F1 generation was similar in all groups.

Achieved dosage:

The achieved dosages for the selected offspring were considered to be satisfactory. As expected, intake of the test substance was higher in these younger animals when compared with their parents but did not suggest that these levels could not be tolerated by the young animals. The mean compound intakes for the F1 animals were approx. 6, 24, 93 and 316 mg/kg bw/day for males and 6, 25, 95 and 313 mg/kg bw/day for females at 50, 200, 750 and 2,500 ppm respectively.

Sexual maturation of the F1 generation:

The timing of balano-preputial separation for the F1 animals was unaffected by treatment. There appeared to be a delay of 3 days in vaginal opening (VO) for females treated at 2,500 ppm when compared to control value ($p < 0.01$); however, this is considered more an incidental finding than a treatment-related effect, particularly when compared to the control value of the definitive two-generation study in rats of 35.0±3.0 days (see [redacted] 2003; M-232532-01-1) and considering the low animal number. Furthermore, no effect on sexual development was observed at 2,000 ppm in the main study. Even if considered treatment-related the slight delay in VO would be assumed secondary to the lower body weights seen in this group rather than a selective effect of treatment.

Table 5.6.1- 15: Sexual development – F1 generation (group mean)

Parameter	Dose level (ppm)				
	0	50	200	750	2,500
Males					
Preputial separation (days)	44±1.9	45±1.8	43±1.7	45±1.0	44±2.2
Body weight at preputial separation (g)	227	228	222	227	210
Females					
Vaginal opening (days)	33±2.0	34±2.1	33±2.1	34±1.4	36±2.9**
Body weight at vaginal opening (g)	111	115	112	118	118

** $p \leq 0.01$ statistically significantly different to controls

Necropsy of F1 animals:

Macroscopic examination at necropsy of the F1 animals at around 7 to 8 weeks of age did not reveal test substance-related findings that were considered to be related to the dietary administration of fluopicolide.

III. Conclusion

This preliminary study showed that dietary concentrations up to 2,500 ppm of fluopicolide induced toxicity observed as body weight gain reductions in F0 in both sexes during the premating period, and in F0 females during the gestation period. A transient effect on bodyweight gain in F0 females was also observed during the gestation period at 750 ppm. F1 offspring body weight gains were also reduced at 2,500 ppm from approx. Day 14 of age. A dose level up to 2,500 ppm would be suitable as the high concentration in a main study of reproductive performance. This concentration would be expected to elicit a measurable degree of parental toxicity without affecting fertility or reproductive performance.

Assessment and conclusion by applicant:

This preliminary study was valid and acceptable to aid in the selection of doses for a subsequent 2-generation study.

Data Point:	KCA 5.6.1/02
Report Author:	[redacted]
Report Year:	2003
Report Title:	Study of reproductive performance in CD rats treated continuously through two successive generations by dietary administration (Volume 1 of 3) Code: AE C638206
Report No:	C033054
Document No:	M-232532-01-1
Guideline(s) followed in study:	JMAFF 12 Nohsan No. 8147 (2000); OECD 416 (1999); US-EPA-OPPTS 870.3800 (1998)
Deviations from current test guideline:	Deviations from the current OECD guideline (416, 2001): None.
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

The influence of fluopicolide on the fertility and reproductive performance of two successive generations was assessed in male and female rats of the Crl: CD® (SD) IGS BR strain. Fluopicolide was administered continuously in the diet at inclusion levels of 0, 100, 500 or 2,000 ppm (parts per million) to groups of rats throughout the two generations.

The F0 generation, which comprised 28 males and 28 females in each group, received the treated diet for 10 weeks before pairing and throughout mating, gestation, littering and lactation. Offspring survival and growth to weaning were evaluated at which point 24 male and 24 female offspring per group were selected to form the F1 generation. Both sexes received similar treated diets as their parents for a minimum of 10 weeks from selection, throughout pairing, gestation, littering and lactation. Sexual maturation, fertility and reproductive capacity of the F1 generation were assessed and the resulting F2 offspring were monitored for survival and development until weaning.

All F0 and F1 adult animals were subjected to a detailed necropsy, the reproductive organs and other selected organs were weighed and retained. Sperm motility, morphology and counts were assessed for all F0 and F1 males in the control and high treatment groups. Histopathological examinations were performed on designated tissues from ten parent males and ten parent females in the control and high dosage groups, and abnormal tissues from all other parental animals. Unselected F1 and F2 offspring were killed on day 34 of age. Where possible, one male and one female from each litter were subjected to a necropsy examination, the reproductive organs retained, and the brain, spleen, and thymus weighed and retained.

The general condition of the F0 and F1 adults was satisfactory throughout the study. There were no treatment-related deaths in either generation for the adult animals. Body weight gain and food consumption were low for adult animals treated at 2,000 ppm throughout the study, with the exception of the low body weight gain which was not apparent in the females following parturition. Food conversion efficiency was considered unaffected. The achieved dosage at all dietary concentrations for both sexes was considered satisfactory and exposure levels in excess of 100 mg/kg bw/day were achieved at 2,000 ppm throughout the treatment period. The intake generally fell to approx. 40 to 50% of the initial values during the course of each pre-pairing period. Achieved intake of the females was generally slightly higher than that of the males. Intake increased as expected for the females during gestation and lactation peaking at around 2.5 to 3 times the pre-pairing value in the second week of lactation.

Oestrous cycles, mating performance, fertility and fecundity were similar in all groups. Gestation length and the parturition process were unaffected by treatment. The return of females to oestrous cycling following lactation was not influenced by treatment in either generation.

Sexual maturation, as assessed by the age and bodyweight at the time of attainment of vaginal opening or balano-preputial separation, was not affected by treatment with fluopicolide at doses up to and including 2,000 ppm.

Litter parameters at birth of the F1 and F2 progeny and their survival to weaning showed no detrimental effects of treatment. Although initial group mean body weight values were similar in all groups, both male and female offspring at 2,000 ppm displayed a similar pattern of significantly reduced body weight from Day 14 through to weaning, coinciding with the time when the offspring started to eat the diet suggesting a palatability effect and/or systemic toxicity due to direct consumption of the test diet.

Macroscopic examination at necropsy of F0 parents, the unselected F1 offspring or the F2 offspring did not indicate any adverse effect of treatment. At 2,000 ppm, kidney and liver weights were high for parental males and females in both generations, when compared with the controls. Group mean body weight-relative liver weights were also slightly higher for females treated at 500 ppm, when compared with the controls. Differences in spleen and/or thymus weights treated at 2,000 ppm were generally attributed to the lower body weights in these groups, although body weight-relative spleen weights were also low for females at this level for all generations.

Sperm analysis in males (control and high treatment groups only) of both generations did not reveal any treatment-related findings.

A retrospective histopathological examination of the liver and kidneys (see [redacted] 2004; M-247289-01-1) showed treatment-related findings in both organs. In the kidneys, treatment-related changes were noted in both sexes from both generations at 2,000 ppm and consisted of cortical tubular basophilia, cortical tubules with hyaline droplets, granular casts in the medulla, hyaline tubular casts, interstitial inflammation and cortical scarring in males and cortical tubular basophilia, cortical tubular dilatation and corticomedullary mineralization in females. In the liver treatment-related changes were present in both sexes at 2,000 ppm and in males at 500 ppm from both generations and consisted of centrilobular hepatocyte hypertrophy.

In conclusion, a dietary concentration of fluopicolide at 500 ppm should be considered as the No Observed Adverse Effect Level (NOAEL), based on the likelihood of the increased liver weights and the slightly increased centrilobular hepatocyte hypertrophy at this dose level being an adaptive change and not an adverse toxicological effect. The minimum mean achieved dosages for the F0 animals at this NOAEL (500 ppm) are 25.5 mg/kg bw/day for the males and 32.9 mg/kg bw/day for the females. The No Observed Effect Level (NOEL) for developing offspring is considered to be 500 ppm, equivalent to a minimum mean achieved dosage of 35.8 mg/kg bw/day for F0 females during gestation and lactation based on the decreased bodyweight gain at 2,000ppm. The NOEL for reproductive parameters is considered to be 2,000 ppm, equivalent to a mean achieved dosage of at least 103.4 mg/kg bw/day for F0 males and at least 127.3 mg/kg bw/day for F0 females before pairing.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 (fluopicolide)
Purity:	95.9%
Batch no.:	OP2050046

2. Vehicle and/or positive control

Vehicle:	Basal diet, no positive control

3. Test animals

Species:	Rat
Strain:	Crl:CD®(SD)IGS BR strain (Sprague-Dawley origin)
Age:	Approximately 6 weeks
Weight at start:	37 to 223 g for the males and 120 to 189 g for the females
Source:	[redacted]
Acclimation phase:	Yes
Diet:	Powdered laboratory animal diet (UAR VFR1 Certified, manufactured by Usine d'Alimentation Rationnelle in France) supplied by Charles River UK, Margate, Kent, England
Water:	Polythene or polycarbonate bottles with sipper tubes
Housing:	The animal room had its own supply of filtered air, which was passed to the atmosphere without recirculation. Depending on the phase of the study, rats were housed in TR18 cages from Arrowmight Biosciences, Hereford, England or RB3 modified and RB3 cages from North Kent Plastic Cages Limited, Erith, Kent, England. The cages consisted of stainless steel (TR18) or high-density polypropylene (RB3 and RB3 modified) bodies with lids of stainless steel grid
Temperature:	19-23 °C
Humidity:	40-70%
Air changes:	Not given (see above)
Photoperiod:	12 hours

B. Study design

1. In life dates: July 11, 2001 to June 19, 2002

2. Animal assignment and treatment:

Upon receipt of the animals, each litter was identified and allocated to cages, a maximum of four littermates of one sex being placed in each cage. Before commencement of treatment, the animals within each sex were weighed and the three litters per sex showing the greatest within-litter variation in body weight were discarded to leave 28 litters of males and 28 litters of females. One animal from each of these litters was randomly allocated to each study group, assigned a number and identified by tail tattoo. This procedure ensured that for each sex, all groups contained populations of rats with a similar genetic background and comparable initial mean body weight and body weight range. No group contained more than one male and one female from the same litter.

The four treatment groups comprising the F0 generation were as follows:

Table 5.6.1- 16: Study design – F0 generation

Test group	Dose level (ppm)	Number of animals	
		Males	Females
1	0	28	28
2	100	28	28
3	500	28	28
4	2,000	28	28

24 males and 24 females per group were selected to form the F1 generation when the F1 offspring were approx. four weeks of age. A minimum of one male and one female per litter were selected from as many litters as possible by using a random number procedure within each available litter.

Where appropriate, additional offspring were selected from randomly selected litters until the required number of animals were selected for the F1 generation. Each selected animal was assigned a number and identified by tail tattoo.

The four treatment groups comprising the F1 generation were as follows:

Table 5.6.1- 17: Study design – F1 generation

Test group	Dose level (ppm)	Number of animals	
		Males	Females
1	0	24	24
2	100	24	24
3	500	24	24
4	2,000	24	24

3. Diet preparation and analysis:

Fresh diet was prepared at Huntingdon Life Sciences, Huntingdon Research Centre at various intervals during the study in batches covering up to two weeks of treatment and prepared up to one week in advance of the first day of use. A pre-mix of a suitable dietary concentration was prepared by adding an approximately equal quantity of plain diet to the required weight of fluopicolide and mixing using a spoon. A further amount of plain diet that approximately equalled this mixture was then added and stirred in with a spoon. This doubling-up procedure was followed until a visibly homogenous pre-mix of the required weight was achieved, and the pre-mix blended in a Turbula Mixer. A second pre-mix was formulated from this first pre-mix using the doubling-up procedure described above and finally blended in a Turbula Mixer.

The 2,000 and 500 ppm formulations were prepared by direct dilution of the first pre-mix with further quantities of plain diet, and the 100 ppm formulation was prepared by direct dilution of the second pre-mix with plain diet. Blending was achieved by mixing in a Turbula Mixer.

Blending in the Turbula Mixer was set at 100 cycles (approx. six minutes duration). Dosages and concentrations were expressed in terms of the test material as supplied.

Information on the homogeneity of mixing, stability and concentration of the test material in the diet was determined by Huntingdon Life Sciences as part of the preliminary study ([redacted] 2002; M-215068-01-0). In that study the homogeneity and stability, during ambient temperature storage for 22 days, were confirmed for fluopicolide in VRF 1 formulation at nominal concentrations of 50 and 2,500 ppm. The storage period represented the maximum time from preparation to completion of use.

In this main study, samples (nominally 200 g) of treated diets were taken at approx. 10-week intervals equivalent to:

a) Start of treatment (week 1 of F0 generation)
b) First week of pairing (week 11 of F0 generation)
c) Week of selection of second generation (week 18 of F0 generation)
d) During pre-pairing phase of second generation (week 8 of F1 generation)
e) Third week of lactation for second generation (week 17 of F1 generation)

These samples were analysed by Huntingdon Life Sciences for test material content using the High Performance Liquid Chromatography assay.

The mean concentrations of fluopicolide in formulations, prepared for use during Weeks 1, 11 and 18 of treatment of the F0 generation and Weeks 8 and 17 of treatment of the F1 generation, ranged from 87.8 to 101% of nominal concentrations and were considered satisfactory.

The test substance was administered at constant concentration (ppm) to the treated animals in their diet, which was available on an ad libitum basis. Males and females of the F0 generation were treated for 10 weeks before pairing and throughout the study until termination. Animals of the F1 generation had access to the same diet as their parents throughout, but the F1 generation was deemed to formally start at nominal Week 4 (approx. 4 weeks of age). They were treated from nominal week 4 for approx. 10 weeks before pairing, and until termination when litters were weaned. The P0 and F1 Control animals received untreated diet from the same batch over the same period.

4. Statistics

Statistical analyses were performed on the majority of data presented and results of these tests, whether significant or non-significant, are presented on the relevant tables. For some parameters, such as mating performance, the similarity of the data was such that analyses were not considered to be necessary.

For data recorded and/or processed by the Xybion computer system for the parental animals, homogeneity of variance was assessed using Bartlett's test. Whenever this was found to be statistically significant a Behrens-Fisher test was used to perform pairwise comparison, otherwise a Dunnett's test was used. Intergroup differences in macroscopic pathology and histopathology were assessed using Fisher's Exact test.

The following sequence of statistical tests was used for body weight and body weight change during gestation and lactation, body weight and body weight change for offspring, food consumption pre-pairing, during gestation and lactation and offspring organ weights:

> If Bartlett's test for variance homogeneity was not significant at the 1% level, then parametric analysis was applied. If the F1 test for monotonicity of dose-response (F1 test) was not significant at the 1% level, Williams' test for a monotonic trend was applied. If the F1 test was significant, suggesting that the dose-response was not monotone, Dunnett's test was performed instead.
>
> If Bartlett's test was significant at the 1% level, then logarithmic and square-root transformations were tried. If Bartlett's test was still significant, then non-parametric tests were applied. If the H1 test for monotonicity of dose-response (H1 test) was not significant at the 1 % level, Shirley's test for a monotonic trend was applied.

Significant differences between Control and treated groups were expressed at the 5% ($p<0.05$) or 1% ($p<0.01$) level.

C. Methods

1. Observations

All animals were observed at least twice daily throughout the study and any visible signs of reaction to treatment were recorded, with details of type, severity, time of onset and duration. In addition a more detailed weekly examination was performed throughout the treatment period.

2. Body weight and food intake

F0 males were weighed on the day that treatment commenced then weekly thereafter. F1 males were weighed at the formal start of the generation, twice weekly until sexual maturation and then weekly thereafter. F0 and F1 females were weighed on the same schedule until pairing and then on Days 0, 6, 13, and 20 after mating, and on Days 1, 4, 7, 14, and 21 of lactation.

Food consumption was recorded on a cage basis (four animals per cage) for F0 and F1 males and females weekly until pairing. Food consumption for females after mating was recorded on an individual basis on Days 0-5, 6-12 and 13-19 after mating and on Days 1-3, 4-6 and 7-13 of lactation.

After Day 14 of lactation, food intake is increasingly influenced by the offspring as they start to eat solid food and is no longer an accurate reflection of maternal intake.

3. Oestrous cycles - pre-pairing, through mating and post-weaning

For 22 days before pairing of the F0 and F1 generations, daily vaginal smears were taken, using cotton swabs, from all females and these were examined to establish the duration and regularity of the oestrous cycle. After pairing with the male, smearing (vaginal lavage) was continued until evidence of mating was observed.

Following weaning, daily vaginal smears were taken from all females on Days 25 to 28 after birth prior to necropsy and used to determine the stage of the oestrous cycle at termination.

Females that failed to litter were killed on day 25 after mating and females experiencing total litter loss were retained until sufficient litters had been reared to eliminate the requirement for a second pairing. In both instances, females were despatched to necropsy without being smeared.

4. Mating

Following the scheduled period of treatment (10 weeks of treatment for the F0 generation; 10 weeks after selection for the F1 generation), males and females from within the same treatment groups were paired on a one-to-one basis for a period of up 3 weeks. If there was no positive indication of mating after 14 days, the male partner was replaced by a proven male from within the same group provided the female had shown evidence of oestrus. Care was taken to avoid pairing siblings.

Each morning following pairing, the trays beneath the cages were checked for ejected copulation plugs and a vaginal smear was prepared from each female and examined for the presence of spermatozoa and the stage of the oestrous cycle. The day on which evidence of mating was found was designated Day 0 of gestation.

Once mating occurred, the males and females were generally separated, and smearing was discontinued. However after inconclusive mating, smearing was continued for up to 5 days to confirm positive mating.

The time elapsing between initial pairing and detection of mating was recorded.

5. Parturition and duration of gestation

From Day 20 after positive evidence of mating, females were inspected three times daily for evidence of onset, progress and completion of parturition. Females from each group were permitted to deliver their young naturally and rear their own offspring until Day 21 of lactation.

The time elapsing between the detection of mating and commencement of parturition was reported as the duration of gestation.

Females were observed daily for evidence of abnormal maternal behaviour.

6. Observations of F1 and F2 litters

All offspring were examined at approx. 24 hours after birth (Day 1 of age) and the following parameters were recorded for each litter:

a) Number of offspring (live and dead)
b) Individual bodyweights of live offspring
c) Sex ratio
d) Observations on individual offspring

Litters were observed daily for evidence of ill health or a reaction to treatment. Daily records were maintained of mortality and consequent changes in litter size. Wherever possible, any offspring found dead were examined externally and internally.

On Day 4 of age, litters containing more than ten offspring were reduced to ten by random culling, leaving, whenever possible, five male and five female offspring in each litter.

The offspring were sexed at weighing and sexes reported for Days 1, 4 (before and after culling) and 21 of age.

Individual F1 offspring were weighed on Days 1, 4 (before culling), 7, 14, 21, 25 and 28 of age.

Some F1 offspring which were selected to form the F1 generation were weighed on Day 27 of age (nominal week 4 - formal commencement of the F1 generation) instead of Day 28. This deviation from the protocol is considered not to have affected the integrity of the study.

Individual F2 offspring were weighed on the same occasions as the F1 offspring however, they were also weighed on Day 21 of age; these data are retained within the raw data but are not presented within the report. Some F2 litters were weighed on Day 26 of age instead of Day 25 in error. This deviation from the protocol is considered to have no impact on the scientific integrity of the study.

On Day 1 of age, the ano-genital distance was measured for all F1 offspring derived from control dams in order to provide background control data. These data have not been presented within the report however, records are held with the raw data.

Sexual maturation of the F1 selected animals was assessed as follows:

Males: Examined daily from Day 38 of age until balano-preputial separation occurred. Body weight was recorded on the day of completion.

Females: Examined daily from Day 28 of age until vaginal opening occurred. Body weight was recorded on the day of vaginal opening.

7. Necropsy, organ weights, histopathology

Euthanasia:

All adult and weaned animals killed at scheduled termination, and offspring (> 14 days of age) that were killed for reasons of animal welfare, were killed by carbon dioxide inhalation.

Offspring culled on Day 4 of age, and any killed in extremis or for humane reasons before Day 14 of age, were killed by an intraperitoneal injection of pentobarbitone sodium. Grossly normal culled offspring were discarded without formal necropsy. Grossly abnormal culled offspring were examined externally and internally and any abnormal tissues retained.

Any parental animals or offspring that were found dead or killed for reasons of animal welfare were subjected to external and internal necropsy examination as soon as possible. Those found dead outside the normal working day were stored in a refrigerator, designated for this purpose, and necropsied the following day.

In the parental F0 and F1 generation, males were killed once the majority of litters had weaned and it had been established that further litters were not required.

Females that littered and reared offspring were killed at Day 28 of lactation after completion of post-weaning vaginal smears. Females that failed to litter were killed on Day 25 after mating and females experiencing total litter loss were retained until sufficient litters had been reared to eliminate the requirement for a second pairing (Day 17/18 of theoretical lactation).

Regarding the offspring (sporadic deaths, unselected F1 offspring and F2 offspring), for early neonates that were found dead, an assessment of the stomach for milk content was made.

Any early neonates that were found dead were retained, where possible, in industrial methylated spirit prior to an external and internal examination. Other offspring that died before scheduled termination were subjected to external and internal examination.

F1 weaned offspring not selected for continuation of the study, were killed on Day 34 of age following completion of the selection process for the next generation. For practical reasons, the majority of offspring from five unselected F1 litters (from females 2F 1147, 3F 1175, 3F 1186, 3F 1187 and 4F 1202), born later than the other litters, were killed before Day 34 of age, however, one male and one female in each of these litters was retained until Day 34 in order to collect comparable organ weights. F2 offspring were all killed on Day 34 of age.

Macroscopic pathology:

All parental animals were subjected to a detailed macroscopic examination for evidence of disease or an adverse reaction to treatment.

The necropsy procedure included a review of the history of each animal, and a detailed examination of the cranial, thoracic, abdominal, and pelvic cavities, and their viscera. The external and cut surfaces of the organs and tissues were examined, either before or after weighing, as appropriate. The number of uterine implantation sites was recorded for the adult females. Abnormalities, interactions and changes were noted, the requisite organs weighed, and the required tissue samples preserved in fixative.

Unselected F1 offspring and all F2 offspring were examined macroscopically for evidence of disease, or adverse reaction to treatment, and for one offspring of each sex per litter (where possible) appropriate organs were weighed and retained. Specimens of any abnormal tissues were also retained.

Sperm analysis (F0 and F1 adult males):

Immediately after sacrifice the left vas deferens, epididymis and testis of each male was removed and the epididymis and testis were weighed and the following sperm parameters determined.

Sperm motility:

A sample of sperm was expressed from the vas deferens into pre-warmed (37 °C) medium M 199 which contained 0.5% w/v bovine serum albumin (BSA Fraction V). A sample for assessment was taken into a 100 µm depth cannula by capillary action and where possible at least 200 sperm per animal analysed using the Hamilton Thorne IVOS Computer Assisted Sperm Analyser (CASA), version 12.00. The percentages of motile and progressively motile sperm were reported.

Sperm morphology:

A 200 µL aliquot of the sperm/medium mixture (described above) was diluted with 800 µL of 4% neutral buffered formaldehyde. After staining with nigrosine and eosin, an air-dried smear was prepared and examined by light microscopy for the assessment of sperm morphology. Where possible at least 200 sperm were assessed for each male. The percentages of normal sperm and major categories of abnormal sperm were reported.

Sperm count:

The left cauda epididymis of each male was weighed, those for the low and intermediate groups (groups 2 and 3) were frozen. For the control and high treatment groups (groups 1 and 4), 10 mL of a mixture of 0.9% saline, 0.01% merthiolate and 0.05% Triton X-100 (SMT) was added and the cauda epididymis was homogenised for at least one minute. An aliquot of this mixture was added to a pre prepared IDENT stain tube (obtained from Microm UK) before being assessed for sperm count using CASA. The concentration (Million/g) and total number of sperm were reported.

Homogenisation-resistant spermatids count:

The left testis of each male of the Control and high treatment groups (groups 1 and 4) was homogenised for at least two minutes in 25 mL of SMT. An aliquot of this mixture was added to a pre-prepared IDENT stain tube before being assessed for homogenisation-resistant spermatid count using CASA. The concentration (Million/g) and total number of spermatids were reported. Testes of the low and intermediate treatment groups (groups 2 and 3) were frozen.

Organ weights:

Parental animals:

The following organs, taken from each F0 and F1 parental animal, were dissected free of adjacent fat and other tissue, and the weights were recorded:

Adrenal glands	Prostate (ventral lobe)
Brain	Seminal vesicles and coagulating gland
Epididymides (L+R)	Spleen
Kidneys	Testes (L+R)
Liver	Thyroid
Ovaries (L+R)	Thymus
Pituitary[+]	Uterus with cervix and oviducts

[+]: weighed post-fixation
L+R: paired organs weighed separately

The weight of each organ was expressed as a percentage of the bodyweight recorded immediately prior to necropsy for all adults surviving to scheduled terminal kill.

Offspring (unselected F1 offspring and F2 offspring):

Where possible the following organs were taken from one male and one female randomly selected from each litter on day 34 of age, dissected free from adjacent fat and other tissue, and the weight recorded:

Brain
Spleen
Thymus

The weight of each organ was expressed as a percentage of the body weight recorded immediately prior to necropsy for each offspring.

Samples of the following tissues were preserved in 10% neutral buffered formalin (NBF), except for the testes and epididymides, which were preserved in Bouin's fluid for at least 24 hours before transfer to 70% industrial methylated spirit:

Parental animals:

Abnormalities*	Pituitary
Adrenal glands	Prostate (ventral lobe)
Brain	Seminal vesicles and coagulating gland
Right epididymis	Spleen
Kidneys	Right testis
Liver	Thyroid
Mammary glands - caudal	Uterus with cervix and oviducts
Ovaries	Vagina

* Preserved for all animals including F1 and F2 offspring

Offspring (F1 offspring and F2 offspring for organ weight determinations):

The offspring randomly selected for organ weights also had the following tissues retained:

Abnormalities*	Spleen
Brain	Testes
Epididymides	Thymus
Ovaries	Uterus with cervix and oviduct
Prostate (ventral lobe)	Vagina
Seminal vesicles and coagulating gland	

* Preserved for all animals including F1 and F2 offspring

Histology

Tissue samples from animals specified in the following table were dehydrated, embedded in paraffin wax, sectioned at four to five micron thickness and stained with haematoxylin and eosin. Testes were stained using a standard PAS method.

Table 5.6.1- 18: Histology

Tissue	Regions to be examined	Special notes
Abnormalities		Examined for all animals including F1/F2 offspring
Adrenal glands		
Epididymis - right	Longitudinal section to allow examination of caput, corpus and cauda	
Mammary glands - caudal		
Ovaries with oviduct	Midline section	5 Sections cut at approximately 100µm (left and right) intervals from the inner third of each ovary to reach the mid-line
Pituitary		
Prostate (ventral lobe) Seminal vesicles and coagulating gland		
Testis (right)		Qualitative evaluation with awareness of stages of spermatogenesis
Uterus with cervix		
Vagina	Transverse section cut at approximately 5 mm from vulva	

Microscopical examination

Parental animals:

Microscopic examination was performed as follows. The tissues specified in the previous table were examined for ten parent males and ten parent females of the control and high treatment groups (groups 1 and 4) sacrificed on completion of the scheduled treatment period and for all adult animals killed or dying before scheduled termination. Mammary glands were retained and examined.

Examination of the ovary of F0 females was limited to a qualitative assessment of the presence of primordial follicles, growing follicles and corpora lutea. For F1 females, in addition to the general qualitative examination of ovarian tissue, a quantitative assessment was made of the primordial follicle population. For this, five sections were cut at about 100 µm intervals from the inner third of each ovary and the primordial follicles manually counted. Primordial follicle counts were limited to 10 females in each of the control and high treatment groups (groups 1 and 4) and females (all groups) that failed to mate or conceive, or that suffered a total litter loss.

A separate, retrospective, histopathological examination of the liver and kidneys (see [redacted] 2004; M-247089-01-1) was additionally performed to determine if there were treatment-related effects in the identified target organs.

II. Results and Discussion

A. Observations:

F0 generation

1. Clinical signs, mortality - F0 generation

The general condition of F0 males and females was satisfactory throughout the generation; no clinical signs were seen that were considered associated with treatment. There were no unscheduled deaths amongst the males, but one female (Animal Number 1176) at 500 ppm was killed in extremis shortly after parturition. This female was found with pale skin and eyes, hunched posture and piloerection having delivered 10 offspring, three of which were dead; of the seven live offspring five still had the placenta attached. All surviving offspring were killed at the same time as the dam. Findings noted at macroscopic necropsy examination were unremarkable. In view of the isolated nature of this death, and in the absence of similar in-life signs being observed for any other animals, it was considered to be a coincidental event unrelated to treatment.

Green staining was observed on the cage tray paper of seven pairs of animals after mating (one pair at 100 ppm, four pairs at 500 ppm and two pairs at 2,000 ppm). In addition green staining was also observed on the cage tray paper of one cage of male animals at 2,000 ppm following their return to the home cage after separation from the females. In view of the sporadic nature of this finding and the absence of similar findings during/after pairing of the F1 generation, this green staining was considered to be of no toxicological significance.

2. Body weight and food intake - F0 generation

Body weight:

In males, at 2,000 ppm group mean body weight and cumulative body weight change values were lower than that of control from the commencement of treatment (Week 1 of treatment, 6% lower than control value, $p < 0.05$) and remained lower throughout the treatment period to termination (Weeks 1-16 of treatment, 7% lower than control value, not statistically significant) with differences frequently attaining statistical significance.

In 500 ppm males initial group mean body weight and cumulative body weight change values were similar to control. From Week 5 of treatment until termination, group mean body weight gain values were occasionally slightly lower or higher than those of the controls. Conversely, therefore due to the lack of a consistent pattern or other supporting data the slight differences in this parameter at this level were considered to reflect normal biological variation. There was also no evidence of an adverse effect of treatment with fluopicolide on group mean body weight or cumulative bodyweight change values in 100 ppm males.

In 2,000 ppm females mean body weight and cumulative body weight change values prior to pairing displayed a similar pattern of lower gains (Weeks 1-10 of treatment, 14% lower than control value, $p < 0.01$) like that observed in the males at this dosage level. Low mean body weight gain (up to 17% lower than control values, $p < 0.01$) continued during the first two weeks of gestation, however, by the end of the gestation period overall body weight change was similar to control, although absolute body weight remained lower (approx. 5% lower than control value). Following parturition, there was no evidence to suggest that treatment with fluopicolide had any noticeable effect on the normal pattern of body weight change associated with the demands of lactation.

At 500 and 100 ppm there was no evidence of an adverse effect of treatment with fluopicolide on body weight or body weight change either prior to pairing, during gestation or during lactation.

An overview is given in Table 5.6.1- 19.

Table 5.6.1- 19: Body weight and body weight gains (g) of parental animals – F0 generation

	Generation	Dose level [ppm]							
		0	100		500		2,000		
Males									
Body weights [g] (*% difference to control*)									
Week 0	**Pre- and postmating**	191	189	*(-1.0)*	188	*(-1.6)*	190	*(-0.5)*	
Week 4		414	410	*(-1.0)*	408	*(-1.4)*	398	*(-3.9)*	
Week 8		532	521	*(-2.1)*	517	*(-2.8)*	498*	*(-6.4)*	
Week 12		573	561	*(-2.1)*	556	*(-3.0)*	539	*(-5.9)*	
Week 16		619	608	*(-1.8)*	603	*(-2.6)*	588	*(-5.0)*	
Body weight gains [g] (*% difference to control*)									
Week 0-4		223	221	*(-0.9)*	220	*(-1.3)*	208	*(-6.7)*	
Week 0-8		341	333	*(-2.3)*	328	*(-3.8)*	308*	*(-9.7)*	
Week 0-12		382	372	*(-2.6)*	367	*(-3.9)*	348*	*(-8.9)*	
Week 0-16		428	419	*(-2.1)*	415	*(-3.0)*	398	*(-7.0)*	
Females									
Body weights [g] (*% difference to control*)									
Week 0	**Premating**	151	155	*(+2.6)*	151	*(±0.0)*	152	*(+0.7)*	
Week 4		241	254	*(+5.4)*	247	*(+2.5)*	232	*(+3.7)*	
Week 8		286	294	*(+2.8)*	283	*(-1.0)*	268	*(-6.3)*	
Week 10		297	309	*(+4.0)*	298	*(+0.3)*	277	*(-6.7)*	
Body weight gains [g] (*% difference to control*)									
Week 0-4		90	99	*(+10.0)*	96	*(+6.7)*	80*	*(-11.1)*	
Week 0-8		134	139	*(+3.7)*	133	*(-0.7)*	116**	*(-13.4)*	
Week 0-10		146	154	*(+5.5)*	148	*(+1.4)*	126**	*(-13.7)*	
Body weights [g] (*% difference to control*)									
GD 0	**Gestation**	300	312	*(+4.0)*	309	*(+3.0)*	283	*(-2.3)*	
GD 6		331	340	*(+2.7)*	335	*(+1.2)*	308*	*(-6.9)*	
GD 13		362	371	*(+2.5)*	366	*(+1.1)*	335**	*(-7.5)*	
GD 20		439	453	*(+3.2)*	449	*(+2.3)*	419	*(-4.6)*	
Body weight gains [g] (*% difference to control*)									
GD 0-6		30	28	*(-6.7)*	26*	*(-13.3)*	25**	*(-16.7)*	
GD 0-13		62	59	*(-4.8)*	57	*(-8.1)*	52**	*(-16.1)*	
GD 0-20		138	141	*(+2.2)*	141	*(+2.2)*	136	*(-1.4)*	
Body weights [g] (*% difference to control*)									
LD 1	**Lactation**	344	357	*(+3.8)*	341	*(-0.9)*	316**	*(-8.1)*	
LD 4		356	373	*(+4.8)*	362	*(+1.7)*	335*	*(-5.9)*	
LD 7		368	382	*(+3.8)*	372	*(+1.1)*	346*	*(-6.0)*	
LD 14		377	394	*(+4.5)*	379	*(+0.5)*	346**	*(-8.2)*	
LD 21		366	370	*(+1.1)*	370	*(+1.1)*	347*	*(-5.2)*	

	Generation	Dose level [ppm] 0	100		500		2,000	
Body weight gains [g] (*% difference to control*)								
LD 1-4		12	16	*(+33.3)*	22**	*(+83.3)*	19**	*(+58.3)*
LD 1-7		24	25	*(+4.2)*	31	*(+29.2)*	30	*(+25.0)*
LD 1-14		33	37	*(+12.1)*	39	*(+18.2)*	31	*(-6.1)*
LD 1-21		22	14	*(-36.4)*	29	*(+31.8)*	31	*(+40.9)*

GD: gestation day; LD: lactation day
* / ** statistically significantly different from control, $p \leq 0.05$ / $p \leq 0.01$

Food intake:

In males, group mean food consumption values at 2,000 ppm were slightly lower than control from Week 1 to 8 prior to pairing (up to 7% lower than the control values, $p < 0.05$) of the F1 generation, after which parity with the control group was attained.

At 500 and 100 ppm, there was no evidence of an effect of treatment on food consumption during the pre-pairing period. It was noted that group mean values at 100 ppm were slightly lower than control. However, this is considered to be a reflection of the slightly lower body weight and body weight gain observed at this dosage level and not a treatment-relationship.

In 2,000 ppm females mean food consumption values during the pre-pairing phase (up to 8% lower than control values, $p < 0.05$) were consistently slightly lower than control values and this pattern continued throughout the gestation (up to 16% lower than control values, $p < 0.01$) and lactation phases (up to 10% lower than control value, $p<0.05$).

There was no evidence of an adverse effect of treatment with fluopicolide on food consumption at 500 or 100 ppm during the pre-pairing, gestation or lactation phases.

An overview is given in Table 5.6.1- 20 to Table 5.6.1- 22.

Table 5.6.1- 20: Food consumption (g/rat/week) prior to pairing – F0 generation

Time period	Dose level (ppm) 0	100		500		2,000	
Males							
Week 1	172	171	*(-0.6)*	169	*(-1.7)*	158**	*(-8.1)*
Week 10	193	197	*(+2.1)*	190	*(-1.6)*	187	*(-3.1)*
Females							
Week 1	127	127	*(±0.0)*	125	*(-1.6)*	115**	*(-9.4)*
Week 10	128	136	*(+6.3)*	136	*(+6.3)*	123	*(-3.9)*

* $p \leq 0.05$; ** $p \leq 0.01$, statistically significantly different from control

Table 5.6.1- 21: Food consumption (g/rat/day) of dams during gestation (g) - F0 generation

Time period	Dose level (ppm) 0	100		500		2,000	
Days 0-5	24	24	*(±0.0)*	25	*(+4.2)*	22**	*(-8.3)*
Days 6-12	27	28	*(+3.7)*	28	*(+3.7)*	25**	*(-7.4)*
Days 13-19	28	29	*(+3.6)*	29	*(+3.6)*	27	*(-3.6)*

* $p \leq 0.05$; ** $p \leq 0.01$, statistically significantly different from control

Table 5.6.1- 22: Food consumption (g/rat/day) of dams during lactation – F0 generation

Time period	Dose level (ppm)						
	0	100		500		2,000	
Days 1-3	34	37	*(+8.8)*	36	*(+5.9)*	35	*(+2.9)*
Days 4-6	50	49	*(-2.0)*	51	*(+2.0)*	48	*(-4.0)*
Days 7-13	68	68	*(±0.0)*	68	*(±0.0)*	60**	*(-13.3)*

* $p \leq 0.05$; ** $p \leq 0.01$, statistically significantly different from control

Food conversion efficiency of males and females before pairing, assessed during the 10 week pre-pairing treatment period, did not indicate any adverse effect of treatment on food utilisation for either sex, with values for each inclusion level being comparable to Control, indicating that food intake and bodyweight/bodyweight gain were in balance.

Achieved test substance doses:

The achieved dosage at all dosage levels for both sexes generally reflected the intended five and four-fold intervals between dietary levels and exposure to fluopicolide was considered to be satisfactory. Exposure levels in excess of 100 mg/kg bw/day were achieved at 2,000 ppm throughout the 10 week pre-mating period and throughout gestation and lactation. The intake fell to approx. 50% of the initial values during the course of the pre-pairing period. Achieved intake of the females was generally slightly higher than that of the males. Intake increased as expected for the females during gestation and lactation peaking at around 2.5 to 3 times the pre-pairing value in the second week of lactation (see Table 5.6.1-23 to Table 5.6.1- 25).

Table 5.6.1- 23: Achieved dosage (mg/kg bw/day) prior to pairing – F0 generation

Time period	Dose level (ppm)							
	Males				Females			
	0	100	500	2,000	0	100	500	2,000
Week 1	-	11.0	55.0	206.4	-	10.7	54.0	199.6
Week 10	-	5.2	25.5	103.4	-	6.4	32.9	127.3

Table 5.6.1- 24: Achieved dosages (mg/kg bw/day) during gestation – F0 generation

Time period	Dose level (ppm)			
	0	100	500	2,000
Days 0-6	-	7.4	38.9	150.3
Days 6-13	-	7.8	39.5	156.6
Days 13-20	-	7.1	35.8	145.4

Table 5.6.1- 25: Achieved dosages (mg/kg bw/day) during lactation – F0 generation

Time period	Dose level (ppm)			
	0	100	500	2,000
Days 1-4	-	10.1	50.6	214.6
Days 4-7	-	13.0	70.1	281.1
Days 7-14	-	17.5	281.1	348.6

3. Reproductive results – F0 generation

Oestrous cycles:

There was no evidence of an adverse effect of treatment with fluopicolide on oestrous cycle regularity at any dosage level investigated.

Pre-coital interval and mating performance:

There was no evidence of an adverse effect of treatment with fluopicolide on mating performance and fertility, as assessed by pre-coital interval, percentage mating, conception rate and fertility index at any dosage level investigated.

Table 5.6.1- 26 gives an overview.

Table 5.6.1- 26: Mating performance and fertility – F0 generation

Parameter	Dose level (ppm)			
	0	100	500	2000
Number mating	28	28	28	28
Number achieving pregnancy	27	26	27	28
Conception rate (%)	96	93	96	100
Fertility index (%)	96	93	96	100

Gestation length, gestation index and parturition:

With the exception of one control female, two females at 100 ppm and one female at 500 ppm, all females were pregnant and gave birth to live offspring.

There was no adverse effect of treatment with fluopicolide on gestation length or gestation index, with the length of the gestation phase being between 22 and 23 days for females in all groups. There were no difficulties evident during the parturition process that were considered to be related to treatment (see Table 5.6.1- 27).

Table 5.6.1- 27: Gestation length and gestation index – F0 generation

Parameter	Dose level (ppm)			
	0	100	500	2000
Number of pregnant animals	27	26	27	28
Number of litters born	27	26	27	28
Gestation index (%)	100	100	100	100
No. of animals with defined gestation length (% of animals)				
Gestation length: 22 days	10 (37)	11 (42)	13 (48)	8 (29)
Gestation length: 22.5 days	11 (41)	7 (27)	9 (33)	11 (39)
Gestation length: 23 days	6 (22)	8 (31)	5 (19)	9 (32)

Offspring results

Litter responses

There were no instances of total litter loss and with the exception of the female at 500 ppm, which was killed in extremis prior to day 1 of lactation, all females reared their litters to maturity. The following assessment is based on 27, 26, 26 and 28 litters surviving to weaning at 0, 100, 500 and 2,000 ppm, respectively.

The general condition of the offspring was similar in all groups and offspring from the test groups showed no adverse clinical findings either while they were being suckled or later as they started to eat the treated diet.

There was no evidence of an adverse effect of treatment with fluopicolide at any dosage level investigated on the number of uterine implantation sites (recorded at termination), litter size at birth, survival of offspring to litter standardisation on Day 4 and subsequent survival to weaning.

Assessment of the sex ratio, from Day 1 to 21 after birth, did not indicate any adverse effects of treatment with fluopicolide upon the survival of either sex throughout this period.

An overview is given in Table 5.6.1- 28.

Table 5.6.1- 28: Uterine implantations, litter data

Parameter	Dose level (ppm)			
	0	100	500	2,000
Litter number	27	26	26	28
Implantations (mean)	14.4	14.7	15.7	14.9
Total litter size on day 1	13.5	14.1	14.6	13.9
Live birth index (%)	98.3	97.8	99.3	98.1
Viability index (%)	98.9	95.9	98.3	99.3
Lactation index (%) – day 7	100.0	99.6	100.0	100.0
Lactation index (%) – day 21	100.0	98.8	99.6	99.6
Sex ratio (day 1):				
Males	6.3	7.0	7.4	7.5
Females	7.1	7.0	7.2	6.3
Sex ratio (day 21):				
Males	4.7	4.7	4.9	5.0
Females	4.9	4.4	5.0	4.8

Offspring body weights (F1 pups)

Although initial group mean body weight values were similar in all groups, both male and female offspring at 2,000 ppm displayed a similar pattern of significantly reduced body weight from Day 14 through to weaning (approx. 8% lower than control values, $p < 0.01$), coinciding with the time when the offspring started to eat the diet suggesting a palatability effect and/or systemic toxicity due to direct consumption of the test diet and not a lactational effect (see also external expert statement below, [redacted] 2018; M-638869-01-1). This is supported by the fact that reduced food consumption and body weight development was observed in all repeated dose rat studies during the initial treatment phase and also in the parental animals of the present reproductive study (see Table 5.6.1- 20).

At 500 and 100 ppm there was no evidence of an adverse effect of treatment with fluopicolide on offspring body weight or body weight change from birth and through to weaning.

An overview is given in Table 5.6.1- 29.

Table 5.6.1- 29: Body weight and body weight gains (g) of F1 pups

Day of Age	Dose level (ppm)					
	0	100		500		2,000	
Males						
Body weights [g] (*% difference to control*)						
Day 1 (before cull)	6.8	6.8	*(0.0)*	6.7	*(-1.5)*	7.0	*(+2.9)*
Day 4 (before cull)	9.5	9.6	*(+1.1)*	9.4	*(-1.1)*	9.4	*(-1.1)*
Day 4 (after cull)	9.6	9.5	*(-1.0)*	9.4	*(-2.1)*	9.4	*(-2.1)*
Day 7 (after cull)	15.7	15.1	*(-3.8)*	15.2	*(-3.2)*	14.8	*(-5.7)*
Day 14 (after cull)	33.0	32.9	*(-0.3)*	32.6	*(-1.2)*	30.4**	*(-7.9)*
Day 21 (after cull)	52.3	52.0	*(-0.6)*	51.5	*(-1.5)*	48.0**	*(-8.2)*
Day 25 (after cull)	70.5	69.8	*(-1.0)*	69.3	*(-1.7)*	64.4**	*(-8.7)*
Day 28 (after cull)	86.7	85.5	*(-1.4)*	85.1	*(-1.8)*	79.3**	*(-8.5)*
Body weight change (g) Day 1-28	79.9	78.7	*(-1.5)*	78.4	*(-1.9)*	72.2**	*(-9.6)*
Females							
Body weights [g] (*% difference to control*)							
Day 1 (before cull)	6.4	6.4	*(0.0)*	6.3	*(-1.6)*	6.6	*(+3.0)*
Day 4 (before cull)	9.1	9.2	*(+1.1)*	8.9	*(-2.2)*	9.0	*(-1.1)*
Day 4 (after cull)	9.1	9.3	*(+2.2)*	8.9	*(-2.2)*	9.0	*(-1.1)*
Day 7 (after cull)	14.9	14.6	*(-2.0)*	14.4	*(-3.4)*	14.2	*(-4.7)*
Day 14 (after cull)	31.9	32.3	*(+1.3)*	31.1	*(-2.5)*	29.4**	*(-7.8)*
Day 21 (after cull)	50.1	50.5	*(+0.8)*	48.8	*(-2.6)*	46.3**	*(-7.6)*
Day 25 (after cull)	66.2	67.2	*(+1.5)*	64.9	*(-2.0)*	61.1**	*(-7.7)*
Day 28 (after cull)	79.4	80.4	*(+1.3)*	77.9	*(-1.9)*	73.8**	*(-7.1)*
Body weight change (g) Day 1-28	73.0	74.0	*(+1.4)*	71.6	*(-1.9)*	67.2**	*(-7.9)*

** $p \leq 0.01$, statistically significantly different from control

Examination results of unselected offspring and F0 parents

Necropsy of offspring

The nature and incidence of findings observed at macroscopic examination of offspring dying before weaning and the unselected offspring killed at weaning, after selection of the F1 generation, did not suggest any adverse effect of treatment with fluopicolide. The majority of the offspring dying before weaning presented with no milk in the stomach. This is a common finding in such offspring and is considered to reflect a possible lack of maternal care. The incidence of pups dying after litter standardisation was negligible and there was no evidence of an increase in mortality associated with transition to treated diet.

Organ weights of unselected F1 offspring:

Both male and female offspring treated at 2,000 ppm displayed slightly lower absolute spleen (Males: 11% lower than control values, $p < 0.05$; Females: 17% lower than control value, $p < 0.01$) and thymus weights (Males: 11% lower than control value, $p < 0.01$; Females: 9% lower than control value, $p < 0.05$) than the concurrent control, largely related to the lower group mean terminal body weight observed at this dosage which is supported by the fact that the body weight-relative organ weight values for male offspring were similar to those of the controls. Similarly, body weight-relative thymus weights for female offspring were considered unaffected, however, body weight-relative spleen values were slightly low (8% lower than control value, $p < 0.05$). Absolute and body weight-relative brain weights were considered unaffected by treatment.

At 500 and 100 ppm there was no evidence of an adverse effect of treatment on absolute or body weight-relative organ weights. Absolute values at 500 ppm tended to be slightly lower than counterpart controls, but they were considered to be within the expected range of biological variation.

An overview is given in Table 5.6.1- 30.

Table 5.6.1- 30: Organ weights of unselected offspring – F1 generation

Time period	Dose level (ppm)							
	Males				Females			
	0	100	500	2,000	0	100	500	2,000
Litter number	27	25	26	28	27	24	26	28
Terminal body weight (g)	128.0	128.1	127.4	118.9**	113.7	114.3	109.1	102.6**
Spleen weight								
Absolute (g)	0.493	0.490	0.454	0.441*	0.402	0.400	0.369	0.334**
Relative (%)	0.3846	0.3839	0.3577	0.3708	0.3523	0.3482	0.3376	0.3255*
Thymus weight								
Absolute (g)	0.544	0.521	0.512	0.486**	0.468	0.477	0.457	0.424*
Relative (%)	0.4265	0.4091	0.4021	0.4101	0.4129	0.4177	0.4215	0.4154

* $p \leq 0.05$; ** $p \leq 0.01$, statistically significantly different from control

Necropsy of F0 parents

Macroscopic necropsy findings for males and females at termination were unremarkable and there was no evidence of an adverse effect of treatment with fluopicolide at any dosage level investigated.

Organ weights F0 parents

In both males and females treated at 2000 ppm, group mean absolute and bodyweight-relative kidney and liver weights were high and spleen weights were low, when compared with the controls; these instances often attained statistical significance, with the most noticeable and consistent difference being recorded for the liver.

An overview is given in Table 5.6.1- 31.

Table 5.6.1- 31: Organ weights of F0 parents

Time period	Dose level (ppm)							
	Males				Females			
	0	100	500	2,000	0	100	500	2,000
Terminal body weight (g)	618.8	605.7	600.0	587.5	325.0	339.2	327.2	305.7*
Liver weight								
Absolute (g)	22.49	23.44	22.54	26.94**	15.58	16.49	16.12	17.84**
Relative (%)	3.628	3.871*	3.756	4.594**	4.800	4.858	4.926	5.828**
Spleen weight								
Absolute (g)	0.908	0.854	0.854	0.800**	0.613	0.634	0.591	0.524**
Relative (%)	0.1473	0.1424	0.1429	0.1369	0.1878	0.1874	0.1804	0.1719*
Kidney weight								
Absolute (g)	4.03	4.01	4.05	4.44*	2.65	2.81	2.63	2.67
Relative (%)	0.654	0.663	0.678	0.757**	0.817	0.829	0.805	0.873**

* $p \leq 0.05$; ** $p \leq 0.01$, statistically significantly different from control

In addition, other inter-group differences in body weight-relative organ weights were observed, however, these were in organs not influenced to the same extent by overall body weight (e.g. the brain, prostate, seminal vesicles, testes, epididymides and thyroids with parathyroid). As the absolute weights of these organs were unaffected, the apparent increases in body weight-relative weights were considered to be an artefact of the adjustment to terminal body weight rather than an effect of treatment.

Absolute and body weight-relative organ weights for animals receiving 500 or 100 ppm were considered unaffected by treatment.

Sperm analysis and morphology of F0 males

Quantitative (Computer Assisted Sperm Analyser) assessment of the sperm parameters (motility, progressive motility, sperm count, homogenisation resistant spermatids) and visual assessment of sperm morphology did not reveal an effect by treatment with fluopicolide.

Marginal reductions in % motile sperm and % normal morphology and increases in the number of decapitate sperm were apparent at 2,000 ppm; however these were accompanied by much larger standard deviations and were attributable to an individual (Animal Number 1104) and these changes did not attain statistical significance. Sperm concentrations and total sperm counts from the cauda epididymis and testis were similar in the control and treated groups (see Table 5.6.1- 32).

Table 5.6.1- 32: Sperm results of F0 males

Parameter	Dose level (ppm)	
	0	2,000
Motile sperm (%)	95±9	90±22
Sperm count (mill/g) (cauda epididymis)	586±159	600±181
Sperm count (mill/g) (testis)	54±12	54±17
Sperm morphology		
Normal (%)	96.9±8.7	94.6±16.6
Decapitate (%)	2.2±8.4	4.3±16.4
Abnormal (%)	0.9±0.7	1.1±0.9

4. Pathology

Histopathology F0 parents:

Microscopic examination of the organs and tissues taken from the F0 males and females did not reveal any obvious findings that were considered to be related to treatment.

A retrospective histopathological examination of the liver and kidneys (see [redacted] 2004; M-247289-01-1) showed treatment-related findings in both organs. In kidneys an increased incidence of a number of degenerative and regenerative changes (cortical tubular basophilia, cortical tubules with hyaline droplets, granular casts in the medulla, hyaline tubular casts, interstitial inflammation and cortical scarring in males and cortical tubular basophilia, cortical tubular dilatation and corticomedullary mineralization in females) were seen at 2,000 ppm. An increased incidence of cortical tubular basophilia and interstitial inflammation was also seen in males and females treated at 500 ppm, respectively. In the liver an increased incidence of centrilobular hepatocyte hypertrophy was seen in males and females at 2,000 ppm and males given 500 ppm. The latter finding is common in the livers of rodents which have been administered xenobiotics, and as such is considered to be an adaptive change and not a toxic effect of treatment.

A mammary gland tumour was recorded in a control female, one female receiving 100 ppm and one female treated at 2,000 ppm. In view of the incidence and distribution of these tumours they were considered to have arisen by chance and be unrelated to treatment with fluopicolide.

Other findings were of a type and severity commonly seen in rats of this age at this laboratory.

Qualitative examination of the primordial follicle population of the F0 females exposed to fluopicolide suggested that they were similar to the controls.

Oestrous cycles at termination F0 females:

Vaginal smears taken post-weaning (days 25 to 28 of lactation) showed that most females had returned to normal oestrous cycles and attained oestrus before termination and, therefore, fluopicolide had no effect on this parameter.

F1 generation

1. Clinical signs, mortality – F1 generation

The general condition of F1 animals was satisfactory throughout the generation; no clinical signs were seen that were considered associated with treatment and there were no unscheduled deaths.

2. Body weight and food intake – F1 generation

Body weight

In males, at the commencement of the F1 generation (at nominal Week 4 of age) group mean body weights at 2,000 ppm were lower (11% lower than control value, $p < 0.01$) than control reflecting the pattern established prior to weaning; values remained consistently lower than those of the controls throughout the generation, frequently attaining statistical significance up to Week 4 (approx. eight weeks of age). However differences in cumulative body weight gain when compared with control were small indicating that, despite lower absolute body weights, a similar pattern of growth occurred, with overall body weight gain values (Day 0-112) being only 4% lower than control.

In 500 and 100 ppm males there was no evidence of an adverse effect on group mean body weight or body weight gain following treatment with fluopicolide. It was noted that at 100 ppm, body weight and body weight gain were slightly lower than control. In the absence of any effect of treatment in 500 ppm males, these values at 100 ppm were considered to be coincidental and reflect the slightly lower group mean body weight of these animals at the start of the male F1 generation.

In 2,000 ppm females mean body weight values were lower than control from the commencement of the female F1 generation throughout the pre-pairing, gestation, and lactation phases (up to 13% lower than control values, $p < 0.01$). Group mean body weight gain values were similar or slightly lower than control up to week 5 of the F1 generation after which some divergence was noted (8% lower than control at the end of the pre-pairing phase: Day 0-70). This pattern of reduced body weight gain (up to 15% lower than control values, $p < 0.05$ or $p < 0.01$) in females continued throughout the gestation phase, however, following parturition, there was no evidence to suggest that treatment with fluopicolide affected the normal pattern of body weight gain during the lactation period with overall gain by weaning being superior to the concurrent control.

In 500 and 100 ppm females there was no evidence of an adverse effect of treatment with fluopicolide on body weight or body weight gain either prior to pairing, during gestation or during lactation.

An overview of the body weight development in males and females in the different periods is given in Table 5.6.1- 33.

Table 5.6.1- 33: Body weight and body weight gains (g) – F1 generation

	Generation	Dose level [ppm]					
		0	100		500	2,000	
Males							
Body weights [g] (*% difference to control*)							
Day 0[#]	**Pre- and postmating**	91	87	*(-4)*	90	*(-1)*	81**	*(-11)*
Day 7		146	139	*(-5)*	144	*(-1)*	132*	*(-10)*
Day 14		205	197	*(-4)*	204	*(±0)*	191	*(-7)*
Day 21		267	259	*(-3)*	265	*(-1)*	250*	*(-6)*
Day 28		327	316	*(-3)*	324	*(-1)*	308	*(-6)*
Day 35		372	361	*(-3)*	369	*(-1)*	353	*(-5)*
Day 42		409	398	*(-3)*	407	*(±0)*	387	*(-5)*
Day 49		441	428	*(-3)*	439	*(±0)*	418	*(-5)*
Day 56		468	453	*(-3)*	465	*(-1)*	442	*(-6)*
Day 63		486	472	*(-3)*	486	*(±0)*	462	*(-5)*
Day 70		503	490	*(-3)*	506	*(+1)*	478	*(-5)*
Day 77		514	500	*(-3)*	516	*(+1)*	486	*(-5)*
Day 84		531	518	*(-2)*	538	*(+1)*	508	*(-4)*
Day 91		546	533	*(-2)*	552	*(+1)*	521	*(-5)*
Day 98		553	539	*(-2)*	557	*(+1)*	525	*(-5)*
Day 105		568	553	*(-3)*	569	*(±0)*	540	*(-5)*
Day 112		580	560	*(-3)*	583	*(+1)*	548	*(-5)*
Body weight gains [g] (*% difference to control*)							
Day 0-7		55	52	*(-5)*	54	*(-2)*	51	*(-7)*
Day 0-14		114	111	*(-3)*	114	*(±0)*	110	*(-3)*
Day 0-49		350	341	*(-3)*	348	*(-1)*	337	*(-4)*
Day 0-84		441	432	*(-2)*	448	*(+2)*	427	*(-3)*
Day 0-112		489	474	*(-3)*	492	*(+1)*	467	*(-4)*

	Generation	Dose level [ppm]					
		0	100		500		2,000	
Females								
Body weights [g] (*% difference to control*)								
Day 0[#]	**Premating**	82	81	*(-1)*	82	*(±0)*	74*	*(-10)*
Day 7		121	123	*(+2)*	122	*(+1)*	112*	*(-7)*
Day 14		159	162	*(+2)*	156	*(-2)*	147*	*(-7)*
Day 21		185	192	*(+4)*	185	*(±0)*	174	*(-6)*
Day 28		211	219	*(+4)*	209	*(-1)*	196*	*(-7)*
Day 35		229	239	*(+4)*	228	*(±0)*	215*	*(-6)*
Day 42		247	258	*(+5)*	243	*(-2)*	227**	*(-8)*
Day 49		259	271	*(+5)*	256	*(-1)*	241*	*(-7)*
Day 56		272	284	*(+4)*	273	*(±0)*	254*	*(-7)*
Day 63		285	292	*(+3)*	285	*(±0)*	265*	*(-7)*
Day 70		297	300	*(+1)*	291	*(+2)*	271**	*(-9)*
Body weight gains [g] (*% difference to control*)								
Day 0-7		40	42	*(+5)*	40	*(±0)*	38	*(-5)*
Day 0-14		77	81	*(+5)*	74	*(-4)*	73	*(-5)*
Day 0-49		177	190	*(+7)*	174	*(-2)*	167	*(-6)*
Day 0-70		215	219	*(+2)*	209	*(-3)*	197*	*(-8)*
Body weights [g] (*% difference to control*)								
GD 0	**Gestation**	298	302	*(+1)*	295	*(-1)*	267**	*(-10)*
GD 6		325	329	*(+1)*	321	*(-1)*	289**	*(-11)*
GD 13		356	358	*(+1)*	352	*(-1)*	317**	*(-11)*
GD 20		440	448	*(+2)*	440	*(±0)*	398**	*(-9)*
Body weight gains [g] (*% difference to control*)								
GD 0-6		27	26	*(-4)*	26	*(-4)*	23*	*(-15)*
GD 0-13		58	56	*(-3)*	57	*(-2)*	50**	*(-14)*
GD 0-20		142	146	*(+3)*	144	*(+1)*	132*	*(-7)*
Body weights [g] (*% difference to control*)								
LD 1	**Lactation**	344	342	*(-1)*	339	*(-1)*	299**	*(-13)*
LD 4		351	357	*(+2)*	347	*(-1)*	309**	*(-12)*
LD 7		362	367	*(+1)*	357	*(-1)*	318**	*(-12)*
LD 14		367	373	*(+2)*	361	*(-2)*	321**	*(-12)*
LD 21		359	364	*(+1)*	355	*(-1)*	320**	*(-11)*
Body weight gains [g] (*% difference to control*)								
LD 1-4		7	15	*(+114)*	8	*(+14)*	9	*(+29)*
LD 1-7		18	25	*(+39)*	18	*(±0)*	18	*(±0)*
LD 1-14		23	31	*(+35)*	22	*(-4)*	22	*(-4)*
LD 1-21		15	22	*(+47)*	16	*(+7)*	21	*(+40)*

GD: gestation day; LD: lactation day
* / ** statistically significantly different from control, $p \leq 0.05$ / $p \leq 0.01$
[#] formal commencement of F1 generation; animals nominal week 4 of age

Food intake

Group mean food consumption values for males at 2,000 ppm were slightly lower than control from Week 1 to 8 prior to pairing (up to 7% lower than the control values, $p < 0.05$) of the F1 generation, after which it was comparable to the control group.

In 500 and 100 ppm males, there was no evidence of an effect of treatment on food consumption during the pre-pairing period. It was noted that group mean values at 100 ppm were slightly lower than control, however this is considered to be a reflection of the slightly lower body weight and body weight gains observed at this dosage level and not a treatment-relationship.

In 2,000 ppm females group mean food consumption values during the pre-pairing phase were consistently slightly lower than control values (up to 8% lower than control values, $p < 0.05$), and this pattern continued throughout the gestation (up to 16% lower than control values, $p < 0.01$) and lactation phases (up to 10% lower than control value, $p < 0.05$).

There was no evidence of an adverse effect of treatment with fluopicolide on food consumption in 500 or 100 ppm females during the pre-pairing, gestation or lactation phases.

An overview of the food uptakes in males and females in the different periods is given in Table 5.6.1-34 to Table 5.6.1- 36.

Table 5.6.1- 34: Food consumption (g/rat/week) prior to pairing – F1 generation

Time period	Dose level (ppm)							
	Males				Females			
	0	100	500	2000	0	100	500	2,000
Week 1 (% control)	118	111	115	110* (93)	100	102	102	99 (99)
Week 10 (% control)	194	191	197	193 (99)	141	142	140	133 (94)

* $p \leq 0.05$; ** $p \leq 0.01$, statistically significantly different from control

Table 5.6.1- 35: Food consumption (g/rat/day) of dams during gestation – F1 generation

Time period	Dose level (ppm)			
	0	100	500	2,000
Days 0-5 (% control)	25	25	25	22** (88)
Days 6-12 (% control)	28	27	27	25** (89)
Days 13-19 (% control)	31	30*	29*	26** (84)

* $p \leq 0.05$; ** $p \leq 0.01$, statistically significantly different from control

Table 5.6.1- 36: Food consumption (g/rat/day) of dams during lactation – F1 generation

Time period	Dose level (ppm)			
	0	100	500	2,000
Days 1-3	42	45	40	38
Days 4-6 (% control)	51	54	49	46* (90)
Days 7-13 (% control)	72	73	69	66* (92)

* $p \leq 0.05$; ** $p \leq 0.01$, statistically significantly different from control

Food conversion efficiency, assessed during the 10 week pre-pairing treatment period, did not indicate any adverse effect of treatment on food utilisation for either sex, with values for each inclusion level being comparable to control, indicating that food intake and body weight/body weight gain were in balance.

Formal treatment of the F1 generation begins at approx. 4 weeks of age compared to 6 weeks of age in the F0 generation. This automatically results in a higher exposure level in the F1 generation provided food intake and body weight gains are not adversely affected.

Achieved test article doses:

The achieved dosage at all dosage levels for both sexes continued to reflect the intended five and fourfold intervals between dietary levels, and exposure to fluopicolide was considered to be satisfactory.

Exposure levels well in excess of 100 mg/kg bw/day were achieved at 2,000 ppm throughout the 10-week pre-mating period and during gestation and lactation. The intake fell to approximately 40 to 50% of the initial values during the course of the pre-pairing period. Achieved intake of the females was generally slightly higher than that of the males. Intake increased as expected for the females during gestation and lactation peaking at around 2.5 to 3 times the pre-pairing value in the second week of lactation.

An overview of the achieved dosages in males and females in the different periods is given in Table 5.6.1- 37 to Table 5.6.1- 39.

Table 5.6.1- 37: Achieved dosage (mg/kg bw/day) prior to pairing - F1 generation

Time period	Dose level (ppm)							
	Males				Females			
	0	100	500	2,000	0	100	500	2,000
Week 1	-	14.1	70.3	296.5	-	14.2	71.6	304.2
Week 2	-	12.2	60.9	247.0	-	12.1	60.1	251.5
Week 3	-	10.8	53.5	221.1	-	10.9	52.8	221.1
Week 10	-	5.7	28.3	117.1	-	6.8	34.6	141.6

Table 5.6.1- 38: Achieved dosages (mg/kg bw/day) during gestation - F1 generation

Time period	Dose level (ppm)			
	0	100	500	2,000
Days 0-6	-	8.0	40.3	158.9
Days 6-13	-	7.9	40.2	162.6
Days 13-20	-	7.3	37.2	147.0

Table 5.6.1- 39: Achieved dosages (mg/kg bw/day) during lactation - F1 generation

Time period	Dose level (ppm)			
	0	100	500	2,000
Days 1-4	-	12.8	58.6	252.2
Days 4-7	-	14.8	69.9	294.1
Days 7-14	-	19.7	96.0	414.9

Sexual maturation:

There was no evidence of an adverse effect of treatment with fluopicolide on the time of completion of vaginal opening or balanopreputial separation at any dosage level investigated (see Table 5.6.1- 40).

Table 5.6.1- 40: Sexual development – F1 generation (group mean ± SD)

Parameter	Dose level (ppm)			
	0	100	500	2,000
Males				
Preputial separation (days)	44.4±1.7	44.6±3.5	45.0±2.1	45.9±2.4
Body weight at preputial separation (g)	219.8	217.4	223.5	220.9
Females				
Vaginal opening (day)	35.0±3.0	34.4±2.1	34.9±1.9	35.2±2.2
Body weight at vaginal opening (g)	116.7	118.5	115.8	111.3

SD: Standard deviation

3. Reproductive results - F1 generation

Oestrous cycles:

There was no evidence of an adverse effect of treatment with fluopicolide on establishment or regularity of oestrous cycles at any dosage level investigated (see Table 5.6.1- 41).

Table 5.6.1- 41: Oestrus cycle - F1 generation

Incidences	Dose level (ppm)			
	0	100	500	2,000
Number of animals	24	24	24	24
Regular 4- or 5-day cycles	17	23	20	17
Irregular cycles	0	0	0	0
Acyclic	7	1	4	7

Pre-coital interval and mating performance:

There was no evidence of an adverse effect of treatment with fluopicolide on mating performance and fertility, as assessed by pre-coital interval, percentage mating, conception rate and fertility index at any dosage level investigated (see Table 5.6.1- 42).

Table 5.6.1- 42: Mating performance and fertility - F1 generation

Parameter	Dose level (ppm)			
	0	100	500	2,000
Number mating	24	24	24	24
Number achieving pregnancy	24	24	23	23
Conception rate (%)	100	100	100	96
Fertility index (%)	100	100	96	96

Gestation length, gestation index and parturition:

With the exception of one female at 2,000 ppm, all females were pregnant and gave birth to live offspring.

There was no adverse effect of treatment with fluopicolide on gestation length or gestation index with the length of the gestation phase being between 22 and 23 days for females in all groups. There were no difficulties evident during the parturition process that were considered to be related to treatment (see Table 5.6.1- 43).

Table 5.6.1- 43: Gestation length and gestation index – F1 generation

Parameter	Dose level (ppm)			
	0	100	500	2,000
Number of pregnant animals	24	24	24	23
Number of litters born	24	24	24	23
Gestation index (%)	100	100	100	100
No. of animals with defined gestation length (% of animals)				
Gestation length: 22 days	13 (54)	14 (58)	17 (71)	9 (39)
Gestation length: 22.5 days	5 (21)	7 (29)	2 (8)	8 (35)
Gestation length: 23 days	6 (25)	3 (13)	5 (21)	6 (26)

Offspring results

Litter responses

Two females experienced total litter loss, one at 500 ppm on Day 4 of lactation and one at 2,000 ppm on Day 3 of lactation. Most coincidental instances of total litter loss in the CD rat occur during this period and the isolated incidences in this study were considered not to be related to treatment with fluopicolide. The following assessment is based on 24, 24, 23 and 22 litters surviving to weaning at 0, 100, 500 and 2,000 ppm, respectively.

The general condition of the offspring was similar in all groups and showed no adverse responses to treatment of the F1 parents.

There was no evidence of an adverse effect of treatment with fluopicolide at any dosage level investigated on the number of uterine implantation sites (recorded at termination), litter size at birth, survival of offspring to litter standardisation on day 4 and subsequent survival to weaning.

Assessment of the sex ratio, from Day 1 to 21 after birth, did not indicate any adverse effects of treatment with fluopicolide upon the survival of either sex throughout this period.

An overview is given in Table 5.6.1- 44.

Table 5.6.1- 44: Uterine implantations, litter data

Parameter	Dose level (ppm)			
	0	100	500	2,000
Litter number	24	24	23	22
Implantations (mean)	15.6	16.1	15.4	15.3
Total litter size on day 1	14.8	15.4	15.0	14.4
Live birth index (%)	98.3	96.5	99.3	99.4
Viability index (%)	98.8	97.1	97.7	97.2
Lactation index (%) – day 7	100.0	98.8	99.6	100.0
Lactation index (%) – day 21	99.2	96.2	98.3	99.1
Sex ratio (day 1):				
Males	6.8	7.4	8.0	7.0
Females	7.9	8.0	6.9	7.4
Sex ratio (day 21):				
Males	5.0	5.0	5.0	5.2
Females	4.9	4.6	4.6	4.7

Offspring body weights (F2 pups)

At 2,000 ppm, F2 offspring of both sexes showed statistically significantly reduced body weight compared to control from postnatal Day 14 onwards. Consequently, cumulative body weight gain (Day 1-28) was reduced by 14% and 11% for males and females respectively.

Body weight and body weight gains of the F2 offspring treated at 500 or 100 ppm were considered unaffected by treatment with fluopicolide.

An overview is given in Table 5.6.1-45.

Table 5.6.1-45: Body weight and body weight gains (g) of F2 pups

	Dose level (ppm)					
	0	100		500		2,000	
Males						
Body weights [g] *(% difference to control)*						
Day 1 (before cull)	6.5	6.5	*(±0)*	6.5	*(±0)*	6.6	*(+2)*
Day 4 (before cull)	9.2	9.1	*(-1)*	9.0	*(-2)*	9.0	*(-2)*
Day 4 (after cull)	9.3	9.1	*(-1)*	9.0	*(-3)*	9.0	*(-3)*
Day 7 (after cull)	14.7	14.7	*(±0)*	14.2	*(-3)*	14.0	*(-5)*
Day 14 (after cull)	31.4	31.3	*(±0)*	30.7	*(-2)*	28.4**	*(-9)*
Day 21 (after cull)	44.9	44.7	*(±0)*	43.8	*(-2)*	38.9**	*(-13)*
Day 25 (after cull)	63.2	61.9	*(-2)*	60.6	*(-4)*	54.9**	*(-13)*
Day 28 (after cull)	78.6	76.2	*(-3)*	75.1	*(-4)*	68.9**	*(-12)*
Body weight gain (g) Day 1-28	72.1	69.7	*(-3)*	68.5	*(-5)*	62.3**	*(-14)*

	Dose level (ppm)							
	0	100		500		2,000		
Females								
Body weights [g] (*% difference to control*)								
Day 1 (before cull)	6.1	6.1	*(±0)*	6.1	*(±0)*	6.2	*(+2)*	
Day 4 (before cull)	8.6	8.6	*(±0)*	8.5	*(-1)*	8.5	*(-1)*	
Day 4 (after cull)	8.7	8.7	*(±0)*	8.5	*(-2)*	8.6	*(-1)*	
Day 7 (after cull)	13.9	14.0	*(+1)*	13.7	*(-1)*	13.4	*(-4)*	
Day 14 (after cull)	29.9	30.2	*(+1)*	29.6	*(-1)*	27.3**	*(-9)*	
Day 21 (after cull)	42.8	43.7	*(+2)*	42.3	*(-1)*	37.7**	*(-12)*	
Day 25 (after cull)	59.5	59.4	*(±0)*	57.4	*(-3)*	53.1**	*(-11)*	
Day 28 (after cull)	72.5	72.0	*(-1)*	69.8	*(-4)*	65.3**	*(-10)*	
Body weight gain (g) Day 1-28	66.5	65.9	*(-1)*	63.7	*(-4)*	59.1**	*(-11)*	

** $p \leq 0.01$, statistically significantly different from control

Examination results of the F2 offspring and F1 parents

Necropsy of offspring

The nature and incidence of findings observed at macroscopic examination of F2 offspring dying before weaning or at scheduled termination (Day 34 of age), did not suggest any adverse effect of treatment with fluopicolide. The majority of the offspring dying before weaning presented with no milk in the stomach. This is a common finding in such offspring and is considered to reflect a possible lack of maternal care. As in the previous generation, there were virtually no deaths after standardisation of litter size on Day 4 of age.

Organ weights of offspring on Day 34 of age

Both male and female offspring treated at 2,000 ppm displayed lower absolute spleen (Males: 19% lower than control value, $p < 0.01$; Females: 19% lower than control value, $p < 0.01$) and thymus weights (Males: 16% lower than control value, $p < 0.01$; Females: 11% lower than control, $p < 0.05$) than the concurrent control at termination, largely related to the lower group mean terminal body weight observed at this dosage. In females, body weight-relative spleen values were slightly low (9% lower than control value, $p < 0.05$), when compare with the controls.

At 500 and 100 ppm there was no evidence of an adverse effect of treatment on absolute or body weight-relative organ weights of male offspring. Female offspring at these dietary concentrations displayed slightly low absolute spleen weights although this was considered to be consistent with the slightly lower group mean terminal body weight observed and not treatment-related.

An overview is given in Table 5.6.1- 46.

Table 5.6.1- 46: Organ weights of unselected offspring – F2 generation

Time period	Dose level (ppm)							
	Males				Females			
	0	100	500	2,000	0	100	500	2,000
Litter number	24	24	23	22	24	24	23	22
Terminal body weight (g)	116.4	111.5	110.9	100.4**	103.2	97.1	97.7	92.3**
Spleen weight								
Absolute (g)	0.439	0.423	0.428	0.356**	0.375	0.331*	0.335*	0.305**
Relative (%)	0.3772	0.3805	0.3842	0.3543	0.3643	0.3423	0.3424	0.3309*

* $p \leq 0.05$; ** $p \leq 0.01$, statistically significantly different from control

Necropsy of F1 parents

Macroscopic necropsy findings for males and females at termination were unremarkable and there was no evidence of an adverse effect of treatment with fluopicolide at any dosage level investigated.

Organ weights F1 parents

In both males and females treated at 2,000 ppm, group mean absolute and body weight-relative liver and kidney weights were higher than those of the controls, the liver being the organ most noticeably and consistently affected. The females in this group also displayed lower absolute spleen weights, but the body weight-relative spleen weights were not significantly different from controls.

Table 5.6.1- 47: Organ weights of F1 parents

Time period	Dose level (ppm)							
	Males				Females			
	0	100	500	2,000	0	100	500	2,000
Terminal body weight (g)	585.5	567.2	587.3	553.6	320.2	327.7	312.7	290.1**
Liver weight								
Absolute (g)	21.88	21.03	22.88	26.73**	15.31	15.78	16.18	17.95**
Relative (%)	3.732	3.704	3.895	4.833**	4.781	4.813	5.184**	6.189**
Spleen weight								
Absolute (g)	0.843	0.789	0.821	0.777	0.665	0.686	0.624	0.567*
Relative (%)	0.1435	0.1392	0.1398	0.1408	0.2071	0.2105	0.1995	0.1954
Kidney weight								
Absolute (g)	3.82	3.81	3.84	4.49**	2.52	2.66	2.52	2.58
Relative (%)	0.623	0.672	0.656	0.811**	0.790	0.812	0.807	0.890**

* $p \leq 0.05$; ** $p \leq 0.01$, statistically significantly different from control

Group mean body weight-relative liver weights were also slightly high (8% higher than control values, $p < 0.01$) for females treated at 500 ppm, when compared with the controls.

In addition, other inter-group differences in body weight-relative organ weights were observed, however these were in organs that were not influenced to the same extent by overall body weight, such as the brain, seminal vesicles and thyroids with parathyroids. In these cases, the absolute values were considered unaffected, therefore, the apparent increase in body weight-relative weight were considered to be attributable to the adjustment to terminal body weight rather than an effect of treatment.

Sperm analysis and morphology of F1 males

The numbers of motile and progressively motile sperm (from the vas deferens) and the numbers of caudal epididymal sperm and testicular spermatids were similar in all groups. In addition, assessment of sperm morphology suggested that fluopicolide had no adverse effects upon spermatogenesis or upon sperm maturation.

Table 5.6.1- 48: Sperm results of F1 males

Parameter	Dose level (ppm)	
	0	2,000
Motile sperm (%)	90±19	90±7
Sperm count (mill/g) (cauda epididymis)	577±148	587±122
Sperm count (mill/g) (testis)	111±35	127±25
Sperm morphology		
Normal (%)	94.1±20.1	97.6±3.9
Decapitate (%)	5.0±20.2	1.3±2.9
Abnormal (%)	0.9±0.9	1.1±1.2

4. Pathology

Histopathology F1 parents:

Microscopic examination of the organs and tissues taken from the F1 males and females did not reveal any obvious findings that were considered to be related to treatment.

A retrospective histopathological examination of the liver and kidneys (see [redacted] 2004; M-247289-01-1) showed treatment-related findings in both organs. In kidneys an increased incidence of a number of degenerative and regenerative changes (cortical tubular basophilia, cortical tubules with hyaline droplets, granular casts in the medulla, hyaline tubular casts, and cortical scarring in males and cortical tubular basophilia, cortical tubular dilatation and corticomedullary mineralization in females) were seen at 2,000 ppm. In the liver an increased incidence of centrilobular hepatocyte hypertrophy was seen in males and females at 2,000 ppm and males given 500 ppm. The latter finding is common in the livers of rodents which have been administered xenobiotics, and as such is considered to be an adaptive change and not a toxic effect of treatment.

Other findings were of a type and severity commonly seen in rats of this age at this laboratory.

Oestrous cycles at termination F1 females:

Vaginal smears taken post-weaning (Days 25 to 28 of lactation) showed that most females had returned to oestrous cyclicity and attained oestrus before termination and, therefore, fluopicolide had no effect on this parameter.

Fluopicolide had no apparent effect on the primordial follicle count in any group tested when compared to the control values.

III. Conclusion

In conclusion, a dietary concentration of fluopicolide at 500 ppm should be considered as the No Observed Adverse Effect Level (NOAEL), based on the likelihood of the increased liver weights and the slighty increased centrilobular hepatocyte hypertrophy at this dose level being an adaptive change and not an adverse toxicological effect. The minimum mean achieved dosages for the F0 animals at this NOAEL (500 ppm) are 25.5 mg/kg bw/day for the males and 32.9 mg/kg bw/day for the females. The No Observed Effect Level (NOEL) for developing offspring is considered to be 500 ppm, equivalent to a minimum mean achieved dosage of 35.8 mg/kg bw/day for F0 females during gestation and lactation based on the decreased bodyweight gain at 2,000 ppm. The NOEL for reproductive parameters is considered to be 2,000 ppm, equivalent to a mean achieved dosage of at least 103.4 mg/kg bw/day for F0 males and at least 127.3 mg/kg bw/day for F0 females before pairing.

Assessment and conclusion by applicant:

The study was conducted in accordance with OECD TG 416 and is valid and acceptable to assess the reproductive toxicity potential of fluopicolide. A NOAEL for parental toxicity of 500 ppm (equivalent to 25.5 and 32.9 mg/kg bw/d in males and females), A NOAEL of 500 ppm for developing offspring (equivalent to 35.8 mg/kg bw/d for females during gestation) and a NOAEL for reproductive parameters of 2000 ppm (equivalent to 103.4 and 127.3 mg/kg bw/d in males and females) were determined from the study.

An additional histopathological examination to the 2-generation rat study is summarized as follows.

Data Point:	KCA 5.6.1/03
Report Author:	[redacted]
Report Year:	2004
Report Title:	AE C638206 Additional Microscopic Examination to a Study of Reproductive Performance in CD Rats Treated Continuously Through Two Successive Generations by Dietary Administration - Volumes 1 and 2
Report No:	B004908
Document No:	M-247289-01-1
Guideline(s) followed in study:	JMAFF 12 Nohsan No 8147 (2000); OECD 416 (1999); US-EPA OPPTS 870.3800 (1998)
Deviations from current test guideline:	Not applicable; additional histopathological investigation
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

The objective of this evaluation was to extend the histopathological examination to target organs (based on other toxicity studies) from all F0 and F1 animals of the 2-generation rat study ([redacted] 2003; M-232532-01-1). In this study the influence of fluopicolide on the fertility and reproductive capacity of two successive generations was assessed in male and female rats of the CD strain by continuous administered via the diet at concentrations of 0, 100, 500 or 2,000 ppm throughout two generations.

In the kidneys treatment-related changes were noted in both sexes from both generations at 2,000 ppm and consisted of cortical tubular basophilia, cortical tubules with hyaline droplets, granular casts in the medulla, hyaline tubular casts, interstitial inflammation and cortical scarring in males and cortical tubular basophilia, cortical tubular dilatation and corticomedullary mineralization in females.

In the liver treatment-related changes were present in both sexes at 2,000 ppm and in males at 500 ppm from both generations and consisted of centrilobular hepatocyte hypertrophy. This finding is common in the livers of rodents which have been administered xenobiotics, and as such is considered to be an adaptive change and not a toxic effect of treatment.

The additional histopathology on this study confirmed treatment-related findings in the kidneys of animals in the F0 and Fl generations treated at 2,000 ppm and in the liver of animals in the F0 and F1 generations treated at 500 ppm or above. The liver change, centrilobular hepatocyte hypertrophy, was considered to be adaptive and not an adverse toxicological effect.

Based on the kidney findings at 2,000 ppm, it is concluded that 500 ppm should be considered as the No Observed Adverse Effect Level (NOAEL) in this study.

Results:

This histopathological examination revealed an increased incidence of centrilobular hepatocyte hypertrophy in F0 males and females given fluopicolide at 2,000 ppm and males given 500 ppm.

In the kidneys, an increased incidence of a number of degenerative and regenerative changes was seen in animals given fluopicolide at 2,000 ppm. An increased incidence of cortical tubular basophilia and interstitial inflammation was seen in males and females treated at 500 ppm, respectively.

An overview is given in Table 5.6.1- 49.

Table 5.6.1- 49: Histopathological findings in the liver and kidney - F0 generation

Findings	Dose level (ppm)							
	Males				Females			
	0	100	500	2,000	0	100	500	2,000
Number of organs examined	28	28	28	28	28	28	27	28
Liver								
Hepatocyte hypertrophy, centrilobular	0	0	9**	28**	0	0	0	12**
Kidney								
Cortical tubular basophilia total	12	16	19	28**	13	10	19	2
Cortical tubules with hyaline droplets total	1	1	0	28**	0	0	0	0
Granular casts, medulla	0	0	1	20**	0	0	0	0
Interstitial inflammation	0	5	9	15*	0	3	5*	2
Cortical scarring	2	2	2	10*	1	2	3	3
Hyaline tubular casts	2	2	4	10*	3	1	3	0
Cortical tubular dilatation	1	0	0	0	0	0	3	12**
Corticomedullary mineralization	0	0	0	1	3	4	3	10

* $p \leq 0.05$; ** $p \leq 0.01$

Also in the F1 generation an increased incidence of centrilobular hepatocyte hypertrophy was seen in animals given fluopicolide at 2,000 ppm and males given 500 ppm.

In the kidneys an increased incidence of a number of degenerative and regenerative changes were seen in animals given fluopicolide at 2,000 ppm.

An overview is given in the following table.

Table 5.6.1- 50: Histopathological findings in the liver and kidney – F1 generation

Findings	Dose level (ppm)							
	Males				Females			
	0	100	500	2,000	0	100	500	2,000
Number of organs examined	24	24	24	24	24	24	24	24
Liver								
Hepatocyte hypertrophy, centrilobular	0	0	8**	24**	0	0	0	20**
Kidney								
Cortical tubular basophilia total	10	11	11	23**	10	7	9	22**
Cortical tubules with hyaline droplets total	0	0	0	22**	0	0	0	0
Granular casts, medulla	0	0	0	14**	0	0	0	0
Cortical scarring	0	2	1	6*	1	1	0	1
Hyaline tubular casts	2	4	5	13**	1	1	1	0
Cortical tubular dilatation	1	0	0	5	1	1	2	11**
Corticomedullary mineralization	0	0	0	0	3	0	5	8

* $p \leq 0.05$; ** $p \leq 0.01$

The additional histopathology of this study confirmed treatment-related findings in the kidneys of animals in the F0 and F1 generations treated at 2,000 ppm and in the liver of animals in the F0 and F1 generations treated at 500 ppm or above. The hyaline droplets in the cortical tubules of male rats are considered likely to represent accumulation of α2µ-globulin within the lysosomes. It is generally regarded that α2µ-globulin nephropathy is a male rat specific toxic response to the administration of certain types of chemicals (hydrocarbon nephropathy). However, there is a clear effect of administration of fluopicolide on the kidney of female animals as well. This indicates that the kidney is a target organ for this compound.

The liver change, centrilobular hepatocyte hypertrophy, was considered to be adaptive and not an adverse toxicological effect.

Based on the kidney findings at 2,000 ppm, it is concluded that 500 ppm should be considered as the No Observed Adverse Effect Level (NOAEL) in this study.

III. Conclusion

The additional histopathology of this study confirmed treatment-related findings in the kidneys of animals in the F0 and F1 generations treated at 2,000 ppm and in the liver of animals in the F0 and F1 generations treated at 500 ppm or above. The liver change, centrilobular hepatocyte hypertrophy, was considered to be adaptive and not an adverse toxicological effect.

Based on the kidney findings at 2000 ppm, it is concluded that 500 ppm should be considered as the No Observed Adverse Effect Level (NOAEL) in this study.

Assessment and conclusion by applicant:

The additional histopathological investigations confirmed the findings of the main 2-generation study, which was considered valid and acceptable.

CA 5.6.2 Developmental toxicity studies

Data Point:	KCA 5.6.2/01
Report Author:	[redacted]
Report Year:	2000
Report Title:	AE C638206 - Code: AE C638206 00 1C99 0005 - Rat oral developmental toxicity (teratogenicity) range finding study
Report No:	C010137
Document No:	M-198488-01-1
Guideline(s) followed in study:	OECD 414 (1981); Commission Guideline 88/302/EWG (1987); US-US 712-C-98-207, OPPTS 870.3700 (1998); JMAFF 59 NohSan No. 4200 (1985)
Deviations from current test guideline:	As a dose-range finding study the study was not intended to comply with OECD guidelines.
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

This range finding study was conducted in order to select suitable doses of fluopicolide for a subsequent developmental toxicity (teratogenicity) study in Sprague Dawley rats.

Groups of 4 mated female Sprague Dawley rats received technical fluopicolide in aqueous methylcellulose (1% w/v) by oral gavage once daily at the dose levels of 500 or 1,000 mg/kg bw from Day 7-20 of pregnancy (Day 0: day of mating, Day 1: day of sperm detection) and were sacrificed on Day 21 of pregnancy.

Animals were observed daily for mortality and clinical signs of toxicity. Body weight and food consumption were determined regularly throughout the study. At necropsy the dams were examined for macroscopically visible changes. The uterus was opened and the number of live and dead foetuses and the number of conceptuses undergoing resorption were determined. Body weights, crown-rump lengths and placental weights were determined.

No deaths occurred throughout the study. Two animals from the 1,000 mg/kg bw/day group showed pultaceous loose faeces from Day 10 up to Day 13 of pregnancy. At 1,000 mg/kg bw/day body weight gains were lower than normal and also compared to the 500 mg/kg bw/day group throughout the treatment period. In addition, food consumption showed a marked initial decrease in this group between Days 7 and 10 and was slightly reduced during this period also at 500 mg/kg bw/day. No compound-related effects were observed at necropsy of the animals.

Incidence of post-implantation loss was increased at 1,000 mg/kg bw/day even after one animal from this group with total litter loss was excluded from the calculations. In addition, mean foetal weight and crown-rump length were reduced at this dose. Slight decreases in foetal weights and foetal crown-rump lengths were also observed at 500 mg/kg bw/day.

Based on the results of this study, the high dose in the main study should be between 500 and 1,000 mg/kg bw/day.

I. Materials and Methods

A. Materials

1. Test material:

Identification:	AE C638206 (fluopicolide)
Batch:	PP/241024/2 & PP241067/1
Purity:	97.6% w/w (T/041/99, dated 18 or 23 August, 1999)

2. Vehicle and/or positive control:

1% (w/v) methyl cellulose in deionised water

3. Test animals

Species:	Rat
Strain:	Hsd: Sprague Dawley SD
Age:	approximately 8-10 weeks
Weight at start:	191 – 215 g
Source:	[redacted]
Acclimation phase:	yes
Diet:	commercial diet for laboratory rats Ssniff R-Z (V1324)2, *ad libitum*
Water:	tap water in plastic bottles, *ad libitum*
Housing:	in fully air-conditioned rooms in Macrolon cages (Type 3) on soft wood granulate
Temperature:	approx. 22 ± 3 °C
Humidity:	approx. 50 ± 20%
Air changes:	16-20 air changes/hour
Photoperiod:	12 hours

B. Study design

1. In-life dates: September 21 to October 12, 1999

2. Animal assignment and treatment:

The test animals were assigned to the following groups (see Table 5.6.2- 1).

Table 5.6.2- 1: Study design

Test group	Dose level [mg/kg bw/day]	Number of females
1	500	4
2	1,000	4

3. Duration of dosing:

Female animals showing sperm in the vaginal smear after mating received fluopicolide at the dose levels of 500 or 1,000 mg/kg bw/day from Day 7-20 of pregnancy.

4. Dose preparation and administration:

Female animals showing sperm in the vaginal smear after mating received fluopicolide at the dose levels of 500 or 1,000 mg/kg bw as an aqueous preparation in 1% methyl cellulose orally by gavage once daily from Day 7-20 of pregnancy. All groups received a dose volume of 5 mL/kg bw, with adjustment of the individual volume to the most recently recorded body weight. The test substance was prepared daily, immediately before dosing.

5. Mating:

Virgin female animals in the pre-oestrus or oestrus phase were mated overnight with sexually mature males in the ratio 1 male : 1 female and were caged individually after detection of sperm in vaginal smears. The day of sperm detection was defined as Day 1 of pregnancy, and the day of mating was defined as Day 0 of pregnancy. Pregnancy was confirmed at necropsy by the detection of implantation sites or normally developed corpora lutea.

6. Statistics:

Due to the low animal number and the lack of an untreated control group no statistical analyses were performed.

C. Methods

1. Observations:

All animals were examined before the start of the study and were shown to be in good general health condition. The behaviour and general health condition of the animals were observed several times daily.

2. Body weights and food consumption:

Body weights were recorded on Days 1, 4, 7, 10, 14, 17-19 and 21 of pregnancy, and food consumption between Days 1-4, 4-7, 7-10, 10-14, 14-17, 17-19 and 19-21.

3. Caesarean section and foetal evaluation:

The animals were killed on Day 21 of pregnancy and the foetuses removed by Caesarean section. All animals were autopsied and checked for macroscopically visible changes, with emphasis on the uterus. Gravid uterus weight was recorded. The live and dead foetuses in the uterus as well as the conceptuses undergoing resorption and corpora lutea were counted and examined macroscopically. The foetuses, the placentae and conceptuses undergoing resorption were removed from the uterus, weighed or measured and examined for gross external abnormalities and then the foetuses were killed by CO_2 asphyxia.

II. Results and Discussion

A. Observations:

1. Maternal data:

No deaths occurred throughout the study. Two animals from the 1,000 mg/kg bw/day group showed pultaceous faeces from Day 10 up to day 13 of pregnancy. Females of the 500 mg/kg bw/day group showed no clinical signs.

2. Body weight and food consumption:

At 1,000 mg/kg bw/day body weight gain was reduced throughout the treatment period (Day 7-21: -34% compared to 500 mg/kg bw/day dose group).

Table 5.6.2- 2: Maternal body weight and body weight gain *(% of 500 mg/kg bw/day group)*

	Dose level [mg/kg bw/day]	
	500	**1,000**
Body weight [g]		
Day 1	199.5	204.7
Day 4	209.0	211.7
Day 7	221.5	224.3
Day 10	228.5	227.3
Day 14	245.5	242.7
Day 17	269.0	262.0
Day 19	300.8	274.0
Day 21	326.3	293.7
Cumulative body weight gain [g] *(% difference to 500 mg/kg bw/day dose group)*		
Day 1-7	22	19.6 *(-11)*
Day 7-21 [a]	104.8	69.4 *(-34)*
Day 1-21 [b]	126.8	89.0 *(-30)*
Gravid uterus weight [g]		
Day 21	63.1	42.5

[a] During treatment
[b] During pregnancy

Food consumption showed an initial decrease at 1,000 mg/kg bw/day after start of treatment between Days 7 and 10 (-16% compared to Day 4-7 value) and was slightly reduced during the same period at 500 mg/kg bw/day (-7% compared to Day 4-7 value). Food consumption was generally lower at 1,000 mg/kg bw/day compared to the 500 mg/kg bw/day dose group.

Table 5.6.2- 3: Food consumption during gestation

	Dose level [mg/kg bw/day]	
	500	**1,000**
Mean Food Consumption [g/animal/day]		
Day 1-4	15.0	13.3
Day 4-7	18.2	19.7
Day 7-10	17.0	16.5
Day 10-14	21.4	21.3
Day 14-17	21.7	21.0
Day 17-19	25.2	19.8
Day 19-21	22.9	20.2
Total [g/animal]	397.4	376.3

3. Necropsy findings:

No compound-related effects were observed at necropsy of the animals.

4. Caesarean section data:

Incidence of post-implantation loss was increased at 1,000 mg/kg bw/day. One animal from this group showed total litter loss and in addition another female had a high incidence of resorptions (75% of implantations). Mean foetal weight and crown-rump length were also reduced at this dose level compared to control values of the main study (foetal weight 3.7 g, crown-rump length 36 mm). Slight decreases in foetal weights and foetal crown-rump lengths were also observed at 500 mg/kg bw/day. Placental weights were comparable in both groups.

Table 5.6.2- 4: Summary of caesarean section parameters

Parameter	Dose level [mg/kg bw/day]	
	500	1,000
No. pregnant / no. mated	4 / 4	4 / 4
No. of dams with resorptions only	0	1#
No. dams with live foetuses	4	3
Mean no. corpora lutea/dam	14.0	17.3
Mean no. implantation sites/dam	13.0	15.0
Pre-implantation loss (% of corpora lutea)	9.1	14.6
Post-implantation loss (% implants)	0.0	31.7
Mean no. of resorptions/dam	0.0	5.7
No of dead foetuses	0.0	0.0
Total no. of live foetuses	52	28
Mean no. live foetuses/dam	13.0	9.3
Sex ratio (% males)	Not determined	
Mean foetal weight (g)	3.1	2.8
Crown-rump length (mm)	34.1	32.6
Placental weight (g)	0.496	0.468

Excluded from all calculations

III. Conclusion

Based on the results of this study the high dose in the main study should be between 500 and 1,000 mg/kg bw/day.

Assessment and conclusion by applicant:

The study is valid and acceptable to aid in the dose selection for the main developmental toxicity study in rats.

Data Point:	KCA 5.6.2/02
Report Author:	[redacted]
Report Year:	2004
Report Title:	AE C638206 Code: AE C638206 00 1C99 0005 Rat oral developmental toxicity (teratogenicity) study
Report No:	C044366
Document No:	M-202155-02-1
Guideline(s) followed in study:	OECD 414 (1981); Commission Guideline 88/302/EEC (1987); US-EPA 712-C-98-207, OPPTS 870.3700 (1998); JMAFF 59 NohSan No. 4200 (1983)
Deviations from current test guideline:	Method: Deviations from current guideline SANCO/3029/99 rev. 4: There is no specificity or linearity data presented and the accuracy and precision data are determined from the test suspension samples in the toxicological study. However, there are 6 samples per concentration level with mean accuracies between 70-110% and RSD ≤ 20%. Considering that this analytical method is validated in support of toxicological cold studies, the method validation is considered fit for purpose. ;Study: The following deviations from the OECD-Guideline 414 (2018) occurred: - dose levels were not set in two- to four-fold intervals - thyroid-related parameters (thyroid gland weight, histopathological assessment of the thyroid gland) were not assessed as required in the newest guideline version - anogenital distance of all live rodent foetuses was not measured as required in the newest guideline version - No blood samples were taken from dams at study termination for assessment of thyroid hormones T4, T3 and thyroid stimulating hormone (TSH) as required in the newest guideline version At the time of the study conduct, the test guideline required only that the intermediate dose should be located geometrically between the low and high dose levels.Therefore, the deviations are considered not to compromise the outcome of the study and the measured parameters are considered sufficient to assess the developmental toxicity of fluopicolide.
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Data Point:	KCA 5.6.2/05
Report Author:	[redacted]
Report Year:	1993
Report Title:	Historical control data for development and reproductive toxicity studies using Crl:CD BR rat
Report No:	M-259312-01-1
Document No:	M-259312-01-1
Guideline(s) followed in study:	not applicable
Deviations from current test guideline:	--
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	not applicable
Acceptability/Reliability:	Yes

Executive Summary:

Groups of 23 mated female Sprague Dawley rats received fluopicolide by oral gavage in 1% methylcellulose once daily at the dose levels of 0, 5, 60 or 700 mg/kg bw from Day 7-20 of pregnancy (Day 0: day of mating, Day 1: day of sperm detection) and were sacrificed on Day 21 of pregnancy. The dosing volume was 5 mL/kg bw.

Behaviour and state of health were observed daily in all groups. Body weight and food consumption were determined regularly throughout the study.

At necropsy the dams were examined for macroscopically visible changes. Gravid uterus weight was recorded. The uterus was opened and the number of live and dead foetuses and the number of conceptuses undergoing resorption were determined. Body weights, crown-rump lengths, sex ratios of the foetuses and placental weights were determined. The foetuses were examined for external, visceral, and skeletal anomalies.

There were no deaths during the study. No clinical signs were observed in any of the animals. Body weights and weight gains were decreased in the animals from the high dose group, especially at the beginning of the treatment period during gestational Days 7-10, a gestational phase which is considered to be highly susceptible to influences regarding skeletal development. These animals showed also a slight initial decrease in food consumption after beginning of treatment. Body weight gains and food consumption remained unaffected by the administration of the test compound in the other dose groups.

Gravid uterus weights were comparable in all groups. No compound-related effects were observed at necropsy.

Mean foetal body weights, crown-rump lengths and placental weights were slightly, but statistically significantly decreased in the high dose group. However, litter size, number of live and dead foetuses as well as sex ratios were unaffected by the administration of the test substance. Incidences of early and late conceptuses undergoing resorption were also not affected by the administration of the test compound up to and including the highest tested dose level of 700 mg/kg bw/day.

Morphological examination of the foetuses revealed one foetus with multiple malformations at the vertebral column and pelvis in the intermediate dose group and one foetus with microphthalmia in the high dose group. These findings are considered to be incidental due to their isolated occurrence.

Foetuses from the high dose group showed increased incidences of minor defects comprising aplastic, dysplastic or fused thoracic vertebral arches, aplastic, dysplastic, fragmented, fused or dislocated vertebral centres, fragmented and displaced sternebrae as well as aplastic, dysplastic, shortened, fused, wavy and/or thickened ribs. However, only a small number of foetuses in single litters was affected and these findings are not considered to have adverse consequences for the foetuses in postnatal life. The observations represent mostly a perturbation of ossification, transient in nature, being resolved as ossification progresses

Signs of retarded development were reflected by increased incidences of ossification of less than two caudal vertebral centres, weakly or non-ossified sternebrae and non-ossified metacarpale 5, metatarsale 5 and phalanx III of 1st to 5th toe at 700 mg/kg bw/day.

In conclusion, oral administration of fluopicolide to the pregnant rat at the dose of 700 mg/kg bw/day caused maternal toxicity as evidenced by decreased body weight gains and slightly decreased food consumption. Mean foetal body weights and crown-rump lengths were also slightly decreased at the highest dose level. In addition, minor defects at the thoracic vertebrae, sternebrae and ribs as well as delayed ossification were observed more frequently at this dose level and are considered secondary to the above described maternal toxicity. Fluopicolide was not teratogenic in this developmental toxicity study in rats.

Fluopicolide did not cause any maternal toxicity or embryotoxicity at the dose of 60 mg/kg bw/day or below. Therefore, with regard to the present study the No Observed Effect Level (NOEL) is 60 mg/kg bw/day for maternal toxicity and for developmental toxicity.

I. Materials and Methods

A. Materials

1. Test material:

Identification:	AE C638206 (fluopicolide)
Batch:	PP/241024/2 & PP241067/1
Purity:	1) 97.6% w/w (T/041/99, dated 23 August 1999) 2) 97.8% w/w (T/060/99, dated 01 December 1999)

2. Vehicle and/or positive control:

1% w/v methyl cellulose in deionised water (tylose slime)

3. Test animals

Species:	Rat
Strain:	Hsd: Sprague Dawley SD
Age:	approximately 8-10 weeks
Weight at start:	mean group weights: 226.6 – 227.7 g
Source:	[redacted]
Acclimation phase:	yes
Diet:	commercial diet for laboratory rats Ssniff R-Z (V1324)2, *ad libitum*
Water:	tap water in plastic bottles, *ad libitum*
Housing:	in fully air-conditioned rooms in Macrolon cages (Type 3) on soft wood granulate
Temperature:	approx. 22 ± 3 °C
Humidity:	approx. 50 ± 20%
Air changes:	16-20 air changes/hour
Photoperiod:	12 hours

B. Study design

1. In-life dates: November 2, 1999 to June 15, 2000

2. Animal assignment and treatment:

The test animals were assigned randomly (computer-generated algorithm) to the following groups (see Table 5.6.2- 5).

Table 5.6.2- 5: Study design

Test group	Dose level [mg/kg bw/day]	Number of females
1	0	23
2	5	23
3	60	23
4	700	23

3. Duration of dosing:

Mated female Sprague Dawley rats received fluopicolide from Day 7-20 of pregnancy (Day 0: day of mating; Day 1: day of sperm detection) and were sacrificed on Day 21 of pregnancy.

4. Dose preparation and administration:

The rats received fluopicolide at the dose levels of 0, 5, 60 or 700 mg/kg bw/day as a suspension in 1% w/v methyl cellulose (in deionised water) orally by gavage once daily from Day 7-20 of pregnancy. All groups received a dose volume of 5 mL/kg bw, with adjustment of the individual volume to the most recently recorded body weight. The test substance was prepared daily, immediately before dosing.

5. Test substance analysis

For each concentration, samples were taken towards the start, middle and end of the dosing period (Day 7 to 20). All samples were stored deep frozen prior to analysis. The samples from start and end of the dosing period were analysed. The achieved concentrations of fluopicolide in aqueous methyl cellulose (1%) directly after preparation and after 4 hour storage at room temperature from different container locations were between 80 and 107% of the nominal concentrations confirming a sufficient test substance content, homogeneity and stability in the dosing solution.

6. Mating:

Virgin female animals in the pre-oestrus or oestrus phase were mated overnight with sexually mature males in the ratio 1 male : 1 female and were caged individually after the detection of sperm in vaginal smears. The day of sperm detection was defined as Day 1 of gestation and the day of mating was defined as Day 0 of pregnancy. Pregnancy was confirmed at necropsy by the detection of implantation sites or normally developed corpora lutea.

7. Statistics:

The statistical evaluation assumes a monotone dose-response relationship. Statistical comparisons of the low dose groups with the simultaneous control group were only carried out if significant effects were detectable in the high dose group. In the univariate analysis, two-sided questions (body weight of dams, relative food consumption, crown-rump length, foetal weight and placenta weight) were generally tested as follows: a two-sided comparison with the high dose group was followed by a one-sided test for the low-dose group. In case of the caesarean section data of the foetuses (crown-rump length, foetal weight and placental weight), multivariate statistics were first of all calculated and used in selecting relevant dose groups. For the individual parameters, sequential comparisons with the high dose group and sequential tests at the 5 % level for the low dose were then conducted.

The t-tests and the test statistics of Wilks are based on common variance estimations for all study groups. For the Wilcoxon test the exact distribution of the meaned ranks was calculated.

In the case of the daily food consumption of the dams, the mean consumption per 100 g body weight was always calculated between two successive measurement times and evaluated by the rank sum test after Wilcoxon. In examining the body weights of the dams, the change in weight was determined in comparison to the initial weight. The univariate evaluation was carried out using t-tests.

The caesarean section data of the foetuses were used to calculate litter mean values.

Multivariate evaluation was carried out using the test statistics of Wilks. In the univariate analysis, t-tests were used.

The number of corpora lutea, implantation sites and live foetuses, and quotas of dead embryonic primordia undergoing resorption in the animals were likewise analysed using one-sided Wilcoxon tests.

The findings obtained at autopsy and at body cross-section and skeletal examination of the foetuses were evaluated separately for the foetuses and for the litters by Jackknife t-test at significance levels of 5%. It was examined whether the relative frequencies of findings in the dose groups deviated from those findings in the control group.

C. Methods

1. Observations:

All animals were examined before the start of the study and were shown to be in good general health condition. The behaviour and general health condition of the animals were observed several times daily (on weekends and public holidays once daily).

2. Body weights and food consumption:

Body weights were determined on Days 1, 4, 7, 10, 14, 17, 19 and 21 of pregnancy, and food consumption was recorded between Days 1-4, 4-7, 7-10, 10-14, 14-17, 17-19 and 19-21 of pregnancy.

3. Caesarean section:

The animals were killed on Day 21 of pregnancy and the foetuses removed by caesarean section. All animals were examined externally and internally (thoracic and abdominal contents) for macroscopically visible changes, with emphasis on the uterus. Gravid uterus weight was determined. The live and dead foetuses in the uterus as well as the conceptuses undergoing resorption and corpora lutea were counted and examined macroscopically. The implantation sites in the uterus were counted after staining with ammonium sulphide.

The foetuses, the placentae and conceptuses undergoing resorption were removed from the uterus, weighed or measured and examined for gross external abnormalities. Then the foetuses were killed by CO_2 asphyxia.

4. Foetal evaluation:

Approx. 50% of the foetuses of each litter were fixed in alcohol, necropsied, sexed and checked for anomalies of the internal organs. The carcasses were placed in a solution of potassium hydroxide for clearing and stained with Alizarin red S (bones) and Alcian blue (cartilage). The skeletons were examined and checked for stage of development and abnormalities with the aid of a stereo-microscope.

Foetuses found dead in the uterus at caesarean section were fixed in alcohol and examined for external and skeletal anomalies. The remaining foetuses were transferred in Bouin's solution, examined for organ anomalies referring to Wilson's slicing technique[27] and sexed.

Visceral and skeletal changes were subdivided into four categories (major defects, minor defects, variations and retardations) based on the severity of the finding and/or the spontaneous incidence of the finding.

[27] Wilson, J.G.: Embryological considerations in teratology. In Teratology: Principles and Techniques (J.G. Wilson, J. Warkany, Ed.), page 251-277. University of Chicago Press, Chicago, IL (1965)

II. Results and Discussion

A. Observations:

1. Maternal data:

No deaths or clinical signs were observed during the whole study.

2. Body weight and food consumption:

Body weights were slightly but statistically significantly decreased in the animals from the high dose group during treatment on Days 10-21. The statistically significant decrease in body weight at Day 4 in the high dose group is considered to be fortuitous, since treatment started on day 7. Body weight gain was also decreased at 700 mg/kg bw/day (-8% during treatment period and -12% during gestation if corrected for gravid uterus weight). Especially at the beginning of the treatment period the body weight gain was markedly reduced by 24% during Day 7-10, a gestational phase which is considered to be highly susceptible to influences regarding skeletal development.

Table 5.6.2- 6: Maternal body weight and body weight gain

	Dose level [mg/kg bw/day]					
	0	5		60		700	
Body weight [g] *(% difference to control)*							
Day 1	227.3	227.7	*(±0)*	226.6	*(±0)*	226.1	*(±0)*
Day 4	235.7	234.8	*(±0)*	234.0	*(-1)*	231.4*	*(-2)*
Day 7	248.4	249.1	*(±0)*	245.9	*(-1)*	245.2	*(-1)*
Day 10	259.2	260.1	*(±0)*	257.1	*(-1)*	253.5*	*(-2)*
Day 14	275.1	275.4	*(±0)*	272.2	*(-1)*	267.5*	*(-3)*
Day 17	301.2	300.6	*(±0)*	295.9	*(-2)*	291.3*	*(-3)*
Day 19	330.1	329.4	*(±0)*	326.1	*(-1)*	319.3*	*(-3)*
Day 21	363.0	366.6	*(+1)*	360.1	*(-1)*	350.5*	*(-3)*
Day 21 [a]	292.54	291.46	*(±0)*	287.99	*(-2)*	284.10	*(-3)*
Cumulative body weight gain [g] *(% difference to control)*							
Day 1-7	21.1	21.4	*(+1)*	19.3	*(-9)*	18.5	*(-14)*
Day 7-10	10.8	11.0	*(+2)*	11.2	*(+4)*	8.2	*(-24)*
Day 10-14	16.0	15.2	*(-5)*	15.1	*(-6)*	14.0	*(-12)*
Day 14-17	26.0	25.2	*(-3)*	23.6	*(-9)*	23.8	*(-8)*
Day 17-19	29.0	28.8	*(-1)*	30.3	*(+4)*	28.0	*(-3)*
Day 19-21	32.9	37.2	*(+13)*	34.0	*(+3)*	31.2	*(-5)*
Day 7-21 [b]	114.6	117.5	*(+3)*	114.2	*(±0)*	105.3	*(-8)*
Day 1-21	135.7	138.9	*(+2)*	133.5	*(-2)*	123.8	*(-9)*
Day 1-21 [a]	65.2	63.8	*(-2)*	61.4	*(-6)*	57.4	*(-12)*
Gravid uterus weight [g] (% difference to control)							
Day 21	70.46	75.14	*(+7)*	72.11	*(+2)*	66.40	*(-6)*

* Significantly different from control
# No statistical analyses were performed.
a Corrected for gravid uterus weight
b Treatment period only

The animals from the high dose group showed a slight initial decrease in food consumption (not statistically significant) after begin of the treatment period (Table 5.6.2- 7).

Table 5.6.2- 7: Food consumption during gestation

	Dose level [mg/kg bw/day]							
	0	**5**		**60**		**700**		
Mean Food Consumption [g/100 g body weight] *(% difference to control)*								
Day 1-4	6.6	6.3	*(-5)*	6.6	*(±0)*	6.3	*(-5)*	
Day 4-7	8.1	8.5	*(+5)*	8.3	*(+2)*	8.5	*(+5)*	
Day 7-10	8.5	8.9	*(+5)*	8.9	*(+5)*	8.3	*(-2)*	
Day 10-14	8.2	8.3	*(+1)*	8.5	*(+4)*	8.5	*(+4)*	
Day 14-17	8.1	7.9	*(-2)*	7.7	*(-5)*	8.0	*(-1)*	
Day 17-19	8.4	8.4	*(±0)*	8.5	*(+1)*	8.3	*(-1)*	
Day 19-21	7.4	7.5	*(+1)*	7.5	*(+1)*	7.5	*(+1)*	
Total [g/animal]	426.8	427.9	*(±0)*	429.2	*(+1)*	416.1	*(-3)*	

Body weight gains and food consumption remained unaffected by the administration of the test compound in the other dose groups.

3. Necropsy findings:

Gravid uterus weights were comparable in all groups (Table 5.6.2- 6). No compound-related effects were seen at necropsy of the animals.

4. Caesarean section data:

One female from the control and intermediate dose group and two females from the low and high dose group each did not become pregnant.

There was no increase in the incidence of the numbers of early and late conceptuses undergoing resorption and dead foetuses at any dose level up to and including 700 mg/kg bw/day. Most of the resorptions had diameters between 2 and 8 mm, indicating that they were early conceptuses undergoing resorption. One dead foetus was observed in the low dose group.

An overview of relevant parameters is given in Table 5.6.2- 8.

Table 5.6.2- 8: Results of gestation and Caesarean section

Parameter	Dose level [mg/kg bw/day]			
	0	5	60	700
No. pregnant / no. mated	22 / 23	21 / 23	22 / 23	21 / 23
No. dams with live foetuses	22	21	22	21
Mean no. corpora lutea/dam	15.5	16.0	14.9	15.1
Mean no. implantation sites/dam	13.8	14.8	14.1	13.3
Pre-implantation loss (% of corpora lutea)	10.77	7.04	5.34	12.50
Post-implantation loss (% implants)	8.18	6.75	4.53	1.55
Mean no. of resorptions/dam	0.86	0.95	0.64	0.24
Early resorptions/dam	0.86	0.90	0.64	0.24
Dead foetuses/dam	0.00	0.05	0.00	0.00

* Statistically significantly different from control

5. Foetal Data:

Litter size as well as number of live and dead foetuses remained unaffected by the administration of the test substance. Sex ratio of the foetuses was also not altered by the administration of the test substance. Mean foetal body weights, crown-rump lengths and placental weights were slightly, but statistically significantly decreased in the high dose group only.

Table 5.6.2- 9: Foetal data *(% difference to control)*

Parameter	Dose level [mg/kg bw/day]			
	0	5	60	700
Total no. of live foetuses	284	291	297	274
Mean no. live foetuses/dam	12.9	13.9	13.5	13.0
Sex ratio (% males)	53.9	47.4	41.2	48.2
Mean foetal weight (g)	3.7	3.7 *(±0)*	3.6 *(-3)*	3.4* *(-8)*
Crown-rump length (mm)	36.2	36.0 *(-1)*	36.1 *(±0)*	34.8* *(-4)*
Mean placental weight (g)	0.57	0.53 *(-7)*	0.53 *(-7)*	0.52* *(-9)*

* Statistically significantly different from control

External, skeletal and visceral examination:

No compound-related effects were observed at external examination of the foetuses scheduled for skeletal examination. In all cases there was no dose-dependency, and statistical examination did not reveal differences between the groups.

Foetal findings including increased incidences of a small number of minor skeletal defects and evidence of retarded development that could be ascribed to treatment were generally only observed in the highest dose group of 700 mg/kg bw/day. Increased incidences of skeletal findings above the historical control data range in the intermediate dose group are attributed to one single foetus (60 L05) with multiple malformations at the vertebral column and the pelvis. Although increased incidences of thoracic and rib findings were also found in the highest dose group, the findings in the one foetus in the intermediate group are considered incidental since only one foetus was affected and the defects were different to the ones observed at 700 mg/kg bw/day (see also Table 5.6.2- 12).

An overview of relevant findings is given in Table 5.6.2- 10.

Table 5.6.2- 10: Selected external and skeletal foetal findings (foetal (litter) incidence in %)

Parameter	Classification	Dose level [mg/kg bw/day]				HCD#
		0	5	60	700	
External examination						
No. of foetuses (litters) examined		136 (22)	141 (21)	144 (22)	132 (21)	3031 (435)
Eye						
- microphthalmia	major defect	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.8 (4.8)	0.0-0.0
Skeletal examination						
No.of foetuses (litters) examined		148 (22)	150 (21)	153 (22)	142 (21)	3031 (435)
Tail/lumbar-, sacral-, caudal vertebra / pelvic girdle						
- tail and vertebra aplasia, ilium & ischium dysplasia bilateral	major defect	0.0 (0.0)	0.0 (0.0)	0.7[a] (4.5)	0.0 (0.0)	0.0-0.5
Thoracic vertebral arches						
- Aplasia, dysplasia, fused, fused with attached rib	minor defect	0.0 (0.0)	0.0 (0.0)	0.7[a] (4.5)	2.8 (14.3)	0.0-0.5
Thoracic vertebral centra						
- aplasia, dysplasia, fused, fragmented, dislocated	minor defect	0.0 (0.0)	0.0 (0.0)	0.7[a] (4.5)	7.0* (28.6)	0.0-1.2
Caudal vert. centra						
- ossification of less than 2	retardation	6.8 (31.8)	34.0* (81.0)	24.2* (72.7)	82.4* (100.0)	11.0-35.3
Sternebrae						
- fragmented, longitudinally displaced	minor defect	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	2.1 (14.3)	0.0-1.4
- non- or weakly ossified	retardation	6.1 (36.4)	20.7* (47.6)	20.9* (63.6)	71.8* (100.0)	12.8-36.3

Parameter	Classification	Dose level [mg/kg bw/day] 0	5	60	700	HCD#
Rib						
- aplasia, dysplasia, shortened, fused, primordium of only 9	minor defect	0.0 (0.0)	0.0 (0.0)	0.7[a] (4.5)	4.2 (14.3)	0.0-0.3
- wavy and/or thickened	minor defect	0.7 (4.5)	0.7 (4.8)	0.0 (0.0)	3.5 (9.5)	0.0-1.5
- extra rib at 7th cervical vertebra - short or normally long – uni- or bilateral	variation	2.0 (9.1)	0.0 (0.0)	0.7 (4.5)	3.5 (14.3)	0.0-0.3
Pectoral girdle						
- scapula shortened and bent costad right	minor defect	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.7 (4.8)	0.0-0.3
Forepaw						
- metacarpal 5 non-ossified bilateral	retardation	4.7 (22.7)	27.3* (66.7)	19.0* (63.6)	71.8* (100.0)	7.1-33.6
Hindpaw						
- metatarsal 5 non-ossified bilateral	retardation	0.7 (4.5)	0.7 (4.8)	1.3 (9.1)	7.0 (28.6)	0.0-5.7
- 1st to 5th toe non-ossified bilateral	retardation	1.4 (9.1)	0.0 (0.0)	0.0 (0.0)	5.6 (23.8)	0.0-2.3

* Statistically different from control, $p \leq 0.05$
\# As presented in study report (two-sided 95% tolerance interval range of HCD (per study) of foetal incidences in % based on 21 studies (3031 foetuses, 435 litters) in Sprague Dawley rats
[a] Attributed to one small foetus (weight 2.7 g and CR 26 mm) with multiple malformations

Major defects:

There was one foetus (60 LO5, 2.7 g) with multiple malformations at the vertebral column and the pelvis in the intermediate dose group and one foetus (90 R04, 2.0 g) with microphthalmia in the high dose group. The latter one might be secondary to a general developmental retardation (foetal weight -46% compared to control) and both findings are considered to be incidental due to their isolated occurrence.

In addition, the microphthalmia incidence was within historical control ranges identified in a publication (Lang, P. L.; 1993; M-259312-01-1)[28]. The data of this document cover studies which were conducted in the years before 1993 which is not too far from the experimental duration of present study which was from November 2, 1999 to June 15, 2000. This overview provided historical control range data for the finding microphthalmia on gestation Day 21 of up to 1.09% for foetal and of up to 8.70% for litter incidences. An overview is given in Table 5.6.2- 11.

[28] Charles River (1993): Historical Control Data for Development and Reproductive Toxicity Studies using the CrI:CD®BR Rat, Compiled by MARTA (Middle Atlantic Reproduction and Teratology Association); Edited by Patricia L. Lang, Ph.D., Consultant in Toxicology, September 1993 (M-259312-01-1).

Table 5.6.2- 11: Historical control data range for microphthalmia on gestation day 20 and 21 according to public literature

Eye: microphthalmia	Foetal incidence (%)			Litter incidence (%)		
	Average	SD	Max.	Average	SD	Max.
Gestation day 20[a]	0.027	0.17	1.68	0.195	1.19	12.00
Gestation day 21[b]	0.028	0.15	1.09	0.310	1.51	8.70

[a] Gestation day 20: total studies: 154, total litters: 3240, total foetuses: 22892
[b] Gestation day 21: total studies: 69, total litters: 1458, total foetuses: 14976

Therefore, the foetal and litter incidences of microphthalmia in the present study are clearly within the historical ranges of the data provided by Charles River[28] for Sprague-Dawley rats.

Minor defects:

The incidence of foetuses and litters with minor defects was clearly elevated in the 700 mg/kg bw/day group. However, by definition, minor defects are non-lethal, generally not detrimental to postnatal development and therefore, are considered not to have adverse consequences for the foetuses in postnatal life in contrast to major defects/malformations. In this case, most minor defects represent a perturbation of ossification which is transient in nature and will be resolved as ossification progresses (see also external expert statement below [redacted] 2018; M-638869-01-1).

Minor defects observed more frequently in the foetuses from the high dose group consisted of aplastic, dysplastic or fused thoracic vertebral arches (0/148, 0/150, 1/153, 4/142), aplastic, dysplastic, fragmented, fused or dislocated thoracic vertebral centres (0/148, 0/150, 1/153, 10/142*), fragmented or longitudinally displaced sternebrae (0/148, 0/150, 0/153, 3/142), aplastic, dysplastic, shortened, fused or primordium of only 9 ribs (0/148, 0/150, 1/153, 6/142) as well as wavy and/or thickened ribs (1/148, 1/150, 0/153, 5/142).

Table 5.6.2- 12: Detailed foetal and litter data for minor skeletal defects

Parameter	Group dose level (mg/kg bw/day)			
	0	5	60	700
Skeletal				
No.of foetuses examined	148	150	153	142
No. of litters examined	22	21	22	21
Cervical vertebra				
- primordium of only 6	-	-	-	77 R01
Thoracic vertebral arches				
- aplasia 10, 11, 12, 13th bilateral	-	-	60 L05	-
- aplasia 2nd or 7th unilateral or 9th bilateral	-	-	-	77 R01, 77 R05 79 R07
- dysplasia 9th bilateral	-	-	60 L05	-
- fused 6th & 7th unilateral	-	-	-	84 R06
- fused 9th with attached rib	-	-	60 L05	-

Thoracic vertebral centra				
- aplasia of 9, 10, 11, 12 and 13th bilateral	-	-	60 L05	-
- aplasia 2nd or 9th	-	-	-	77 R05 79 R07
- dysplasia 8th	-	-	60 L05	-
- fragmented 6,7,11, 12, 13th (and/or)	-	-	-	71 R07 74 R03 77 R01 84 R02, 84 R04 88 L03
- fused 6 & 7th	-	-	-	77 R01
- dislocated 8th	-	-	60 L05	-
- dislocated 6th or 11th	-	-	-	84 L05, 84 R06
Sternebrae				
- fragmented and/or longitudinally displaced 4th	-	-	-	77 R05 79 R07 88 R06
Rib				
- anlage of only 9 bilateral	-	-	60 L05	-
- aplasia 2nd, 7th or 9th unilateral	-	-	-	77 R01, 77 R05 79 R07
- dysplasia 9th unilateral	-	-	60 L05	-
- fused 8 & 9th proximal part, unilateral	-	-	-	79 R07
- 13th shortened unilateral	-	-	-	75 L05, 75 R02, 75 R04
- wavy 9th, 10th ,11th (and/or)	14 R07	43 L06	-	79 L01, 79 R03
- thickened 3,4,5,6, 7, & 9, 10, 11th (and/or)	-	43 L06	-	79 L01, 79 L03, 79 R03, 79 R07 89 R07
Pectoral girdle				
- scapula shortened and bent costad right unilateral	-	-	-	79 R03

Because individual foetuses sometimes had more than one minor skeletal defect the overall incidence of affected animals was low (foetal incidence: 18/142; litter incidence 8/21). Of the 34 minor skeletal defects listed in Table 5.6.2- 12, 62% can be ascribed to only two litters (77 and 79). Therefore, the level of concern regarding the increased incidences of minor defects is considered low (ECETOC Monograph 31, 2002)[29].

Moreover, the elevation in minor skeletal defects was correlated with the lower mean foetal body weight and the lower dam body weight gain during gestation and is thus considered mainly caused by general retardation secondary to the maternal toxicity induced at 700 mg/kg bw/day (see Figure 5.6.2- 1 and Figure 5.6.2- 2).

[29] ECETOC. 2002. Guidance on Evaluation of Reproductive Toxicity Data. Monograph No.31

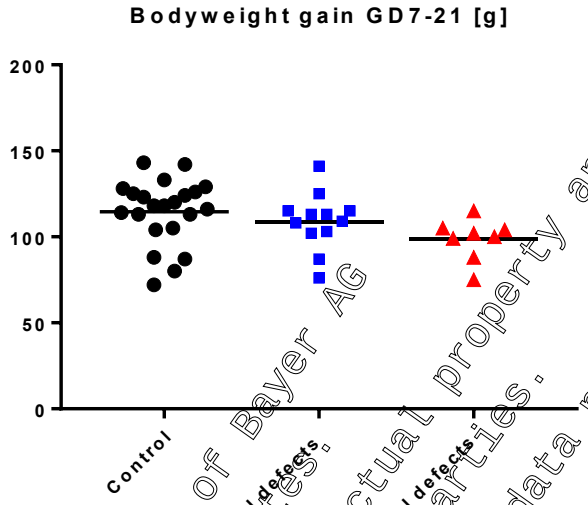


Figure 5.6.2- 1: **Maternal body weight gain (GD7-21) of the control and the high dose group (HD) split into dams that had litters without minor skeletal defects and dams with litters with minor skeletal defects (individual values and mean)**

If the dams of the high dose group are grouped into dams with litters showing minor skeletal defects and dams that had litters without minor skeletal defects it is clearly visible that there is a correlation between dam body weight gain and the occurrence of minor skeletal defects in the litter (Figure 5.6.2-1). The body weight gain of the high dose dams with litters without minor skeletal defects was 109 g compared to 115 g of the control dams (95% of control) whereas the body weight gain of the dams with litters with minor skeletal defects was only 99 g (86% of control).

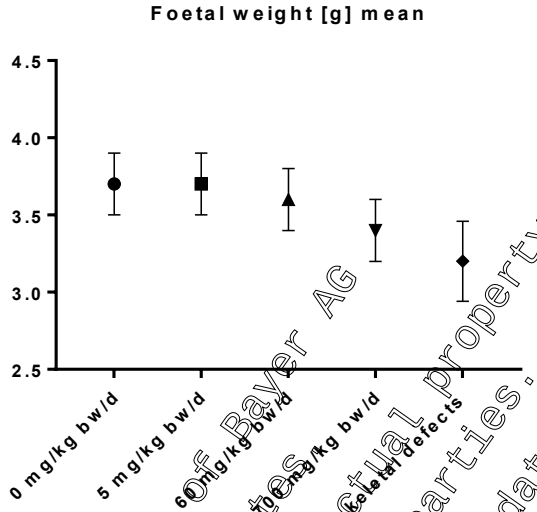


Figure 5.6.2- 2: **Mean foetal weight of control and treatment groups plus foetal weight of foetuses with minor skeletal defects of the highest dose group (mean ± SD).**

Secondary to maternal toxicity the mean foetal body weight at the highest dose level was statistically significantly decreased by 8% compared to control. If only taken into account the foetuses with minor skeletal defects the weight difference was even higher (-14%; see Figure 5.6.2- 2).

Both figures support the hypothesis that the above described skeletal findings are correlated with the lower mean foetal body weight and the lower dam body weight gain during gestation observed at the highest dose level.

Statistical evaluation did not reveal any other skeletal or visceral differences between the groups, and all other incidences were within or only slightly above the historical tolerance limit. Therefore, a compound-related effect is not evident. No abnormalities were detected by examination of cartilage.

Variations

The incidence of an extra rib at the 7th cervical vertebra (3/148, 0/150, 1/153, 5/142) was above the upper limit of the historical confidence interval in the high dose group. However, the value of the control group was also above the HCD range, the difference between control and high dose group was low, and statistical evaluation did not reveal differences between the groups. Therefore, a compound-related effect is questionable. In all other cases the incidences were within or slightly above the historical tolerance limit. Additionally, statistical evaluation did not reveal differences.

Retardations:

Statistical evaluation revealed significant increases in the incidence of ossification of less than 2 caudal vertebral centres (10/148, 51/150*, 37/153*, 117/142*), non- or weakly ossified sternebrae (9/148, 31/150*, 32/153*, 102/142*) and non-ossified metacarpale 5 of the forepaw (7/148, 41/150*. 29/153*, 102/142*) in the foetuses from the high dose group. The incidence of non-ossified metatarsale 5 of the hindpaw (1/148, 1/150, 2/153, 10/142) and of non-ossified phalanx III of 1st to 5th toe of the hindpaw (2/148, 0/150, 0/153, 8/142) was also increased in this group. Since the incidences in the high dose group were distinctly higher than the historical tolerance limit, these changes are considered to be related to treatment and indicate a general developmental retardation of foetal development at this dose level.

Concerning the statistical significant changes in the low and intermediate dose group, the incidences were well within the historical tolerance interval, whereas the control incidences were below the historical control data range (see Table 5.6.2- 10). Additionally, there was no dose-dependency. Therefore, the increased number of retardations observed in the low and intermediate dose groups is considered not to be treatment-related and only statistically different to control because of unusual high ossification grade of control foetuses.

All other findings were within the historical tolerance interval, and statistical evaluation did not reveal differences between the groups

Findings in dead foetuses:

The dead foetus in the low dose group was stunted and showed weak or absent ossification of several bones. No major defects were observed.

III. Conclusion

Oral administration of fluopicolide to the pregnant rat at 700 mg/kg bw/day caused maternal toxicity as evidenced by decreased body weight gain and slightly decreased food consumption especially at the beginning of treatment during gestational Days 7-10, a gestational phase which is considered to be highly susceptible to influences regarding skeletal development. Mean foetal body weights and crown-rump lengths were also slightly but statistically significantly decreased at this dose level. In addition, minor defects at the thoracic vertebrae, sternebrae and ribs as well as delayed ossification considered secondary to the above described maternal toxicity were observed more frequently in the foetuses at this high dose level. Fluopicolide was not teratogenic in this prenatal developmental toxicity study in rats and did not cause any maternal toxicity or embryotoxicity at 60 mg/kg bw/day or below.

With regard to the present study the No Observed Effect Level (NOEL) is 60 mg/kg bw/day for maternal and developmental toxicity.

Assessment and conclusion by applicant:

The study was conducted according to OECD TG 414 and is valid and acceptable to assess the potential of fluopicolide to cause developmental toxicity. A NOAEL of 60 g/kg bw/d was determined from this study for both maternal and developmental toxicity.

Data Point:	KCA 5.6.2/03
Report Author:	[redacted]
Report Year:	2000
Report Title:	Rabbit oral developmental toxicity (teratogenicity range finding study) - AE C638206 - Code: AE C638206 00 1C99 0005
Report No:	C021432
Document No:	M-211192-01-1
Guideline(s) followed in study:	OECD 414 (1981; Commission Guideline 88/302/EEC (1987); US-EPA 712-C-98-207, OPPTS 870.3700 (1998); JMAFF 59 NohSan No. 4200 (1985)
Deviations from current test guideline:	As a dose-range finding study the study was not intended to comply with OECD guidelines.
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

The present study was conducted in order to select suitable dose levels of fluopicolide for a subsequent developmental toxicity study in Himalayan rabbits.

Groups of 4 mated female Himalayan rabbits received technical fluopicolide suspended in 1% (w/v) aqueous methyl cellulose by oral gavage once daily at the dose levels of 25, 50, 100, 250, 500 or 1,000 mg/kg bw from Day 6-28 of pregnancy (Day 0: day of mating) and were sacrificed on Day 29 of pregnancy.

Animals were observed daily for mortality and clinical signs of toxicity. Body weight and food consumption were determined regularly throughout the study.

At necropsy the dams were examined for macroscopically visible changes. The uterus was opened and the number of live and dead foetuses and the number of conceptuses undergoing resorption were determined. Gravid uterus weight was recorded. Foetal body weights, crown-rump lengths and placental weights were determined.

All animals from the 100, 250, 500 or 1,000 mg/kg bw/day group were found dead, killed moribund or killed after abortion up to Day 23 of the study. These animals showed non-specific symptoms including impairments of motility and consciousness, decreased defaecation and hay consumption, hyperactivity and discoloured urine.

At the dose of 50 mg/kg bw/day one animal showed decreased defaecation and discoloured tray. This animal aborted on Day 29. The other animals of the 50 mg/kg bw/day dose group did not show any clinical signs of toxicity. No clinical signs of toxicity were observed at 25 mg/kg bw/day.

Body weight loss and markedly reduced food consumption was observed in animals at dose levels of 100, 250, 500 or 1,000 mg/kg bw/day after start of treatment (Day 6) until day of death/sacrifice of all animals. At 50 mg/kg bw/day, body weight gain and food consumption was reduced throughout the treatment period compared to the 25 mg/kg bw/day dose group animals. Slightly reduced food consumption was also noted in the low dose (25 mg/kg bw/day) animals during the treatment period compared to pre-treatment values.

Necropsy findings in the animals found dead, killed moribund or killed after abortion consisted of beige discoloured heart, liver and kidney. In most animals the stomach showed petechial bleedings and in some was filled with feed mash. No macroscopically visible changes were observed at necropsy of the animals from the 25 and 50 mg/kg bw/day group.

One animal at 25, 50 and 250 mg/kg bw/day were not pregnant. The animal of the 50 mg/kg bw/day group which aborted had six dead foetuses. No abnormalities were observed at caesarean section in the remaining animals from these groups. Gravid uterus and foetal weights were normal and embryofoetal development was unaffected.

Based on the results of this study, a dose level in the region of 50 mg/kg bw/day was considered to be a suitable high dose for the main study.

I. Materials and Methods

A. Materials

1. Test material:

Identification:	AE C638206
Batch:	PP/241024/2 & PP/241067/1
Purity:	97.6 - 97.8% w/w (T/041/99, dated 23 August, 1999, T/060/99, dated 01 December, 1999)

2. Vehicle and/or positive control:

1% (w/v) methyl cellulose in deionised water

3. Test animals

Species:	Rabbit
Strain:	Chbb:HM(SPF) Kleinrusse
Age:	approximately 5-10 months
Weight at start:	2314-2949 g
Source:	[redacted]
Acclimation phase:	yes
Diet:	commercial diet for laboratory rabbits Ssniff K-H (V2333) ad libitum in food racks, additionaly 40-50 g hay daily
Water:	Ad libitum from automatic dispensers
Housing:	in fully air-conditioned rooms in V2A-steel-cage typ: HD3 Fa. Hulskamp;
Temperature:	approx. 22 ± 3°C
Humidity:	approx. 50 ± 20%
Air changes:	16-20 air changes/hour
Photoperiod:	12 hours

B. Study design

1. In-life dates: September 23, 1999 to February 16, 2000

2. Animal assignment and treatment:

The test animals were assigned to the following groups (see Table 5.6.2- 13).

Table 5.6.2- 13: Study design

Test group	Dose level (mg/kg bw)	Number of females
1	25	4
2	50	4
3	100	4
4	250	4
5	500	4
6	1,000	4

3. Duration of dosing:

Female animals showing sperm in the vaginal smear after mating received fluopicolide orally by gavage once daily from Day 6-28 of pregnancy.

4. Dose preparation and administration:

Female animals showing sperm in the vaginal smear after mating received fluopicolide at the dose levels of 25, 50, 100, 250, 500 or 1,000 mg/kg bw/day as an aqueous preparation in 1% methyl cellulose orally by gavage once daily from Day 6-28 of pregnancy. All groups received a dose volume of 5 mL/kg bw, with adjustment of the individual volume to the most recently recorded body weight. The test substance was prepared daily, immediately before dosing.

5. Mating:

Virgin female animals were mated with sexually mature males in the ratio 1 male : 1 female and were caged individually after the detection of sperm in vaginal smears. The day of mating is defined as Day 0 of pregnancy. Pregnancy was confirmed at necropsy by the detection of implantation sites or normally developed corpora lutea.

6. Statistics:

Due to the low animal number and the lack of an untreated control group no statistical analyses were performed.

C. Methods

1. Observations:

All animals were examined before the start of the study and were shown to be in good general health condition. The behaviour and general health condition of the animals were observed several times daily.

2. Body weights and food consumption:

Body weights were recorded on Days 0, 3, 6, 8, 10, 13, 16, 19, 23, 26 and 29 of pregnancy, and food consumption between Days 0-3, 3-6, 6-8, 8-10, 10-13, 13-16, 16-19, 19-23, 23-26 and 26-29.

3. Caesarean section and foetal evaluation:

The animals are killed by intravenous injection of T61® (Hoechst) on Day 29 of pregnancy and the foetuses removed by Caesarean section. All animals were autopsied and checked for macroscopically visible changes, with emphasis on the uterus. Gravid uterus weight was determined. The live and dead foetuses in the uterus as well as the conceptuses undergoing resorption and corpora lutea were counted and examined macroscopically. The foetuses, the placentae and conceptuses undergoing resorption were removed from the uterus, weighed, or measured and examined for gross external abnormalities. The foetuses were killed by CO_2 asphyxia and the crown-rump length was recorded.

II. Results and Discussion

A. Observations:

1. Maternal data:

All animals from the 100, 250, 500 or 1,000 mg/kg bw/day group were either found dead, killed moribund or killed after abortion up to Day 23 of the study.

In detail, at 1,000 mg/kg bw/day, animals were found dead on Day 13 (2 of 4 animals), 14 (1 of 4 animals) and 15 (1 of 4 animals). At 500 mg/kg bw/day, one animal was found dead on Day 15 and the remaining three animals were found dead on Day 16. Two animals of the 250 mg/kg bw/day dose group were found dead on Day 18 and 21, whereas two additional animals were killed moribund on Day 17 and 23. At 100 mg/kg bw/day, two animals were found dead (Day 16 and 20), one animal was killed moribund on Day 22 and another animal was killed after abortion on Day 22.

Animals found dead, killed moribund or killed after abortion showed non-specific symptoms including impairment of motility and consciousness, respiratory sounds, coat bristling, decreased defaecation and hay consumption, tray bedding discoloured, hyperactivity, hypoactivity and discoloured urine.

At the dose of 50 mg/kg bw/day one animal showed decreased defaecation and discoloured tray. This animal aborted on Day 29. The other animals in the 50 mg/kg bw/day dose group did not show any clinical signs of toxicity. No clinical signs of toxicity were observed at 25 mg/kg bw/day.

Table 5.6.2- 14: Maternal mortality data until study termination

	Dose level [mg/kg bw/day]					
	25	**50**	**100**	**250**	**500**	**1,000**
Total animal number per group	4	4	4	4	4	4
Maternal mortality						
No. of animals **found dead**	0	0	2	2	4	4
Day(s) of death/sacrifice	-	-	16, 20	18, 21	15-16	13-15
No. of animals **killed moribund**	0	0	1	2	0	0
Day(s) of death/sacrifice	-	-	22	17, 23	-	-
No. of animals **killed after abortion**	0	1	1	0	0	0
Day(s) of death/sacrifice	-	29	22	-	-	-
Total number animals dead/sacrificed before study termination on day 29	0	0	4	4	4	4

2. Body weight and food consumption:

Mean body weights were impaired in animals at dose levels of 100, 250, 500 or 1,000 mg/kg bw/day after start of treatment (Day 6) until day of death/sacrifice of all animals (Day 22, Day 23, Day 16 and Day 15, respectively).

At 50 mg/kg bw/day, body weight gain was reduced (-57%) throughout the treatment period especially in the one animal that aborted on day 29 compared to the mean body weight gain of the 25 mg/kg bw/day dose group animals.

Table 5.6.2- 15: Mean maternal body weight and body weight gain[#]

	Dose level [mg/kg bw/day]					
	25	**50**	**100**	**250**	**500**	**1,000**
Body weight [g]						
Day 0	2750.3	2471.3	2676.0	2599.0	2763.3	2613.5
Day 3	2807.7	2524.3	2746.3	2554.3	2813.8	2681.8
Day 6	2781.3	2517.3	2725.6	2618.7	2794.0	2695.3
Day 8	2778.0	2543.3	2725.0	2640.0	2724.0	2640.3
Day 10	2788.3	2539.3	2709.8	2630.7	2723.8	2604.3
Day 13	2790.3	2567.7	2629.0	2574.0	2669.5	2496.5
Day 16	2837.0	2603.7	2522.8	2507.0	2578.0	-
Day 19	2831.0	2612.7	2381.3	2351.0	-	-
Day 23	2868.3	2599.3	-	-	-	-
Day 26	2934.0	2641.7	-	-	-	-
Day 29	3002.0	2612.3	-	-	-	-

	Dose level [mg/kg bw/day]					
	25	50	100	250	500	1,000
Cumulative body weight gain [g]						
Day 0-6	31.0	46.0	49.5	19.7	30.7	8.8
Day 6-29	220.7	95.0	-	-	-	-
Day 0-29	251.7	141.0	-	-	-	-

Non-pregant dams were excluded from the means
- No data available due to mortality rate of 100 % of pregnant dams in the respective groups

Reduced mean food consumption was observed in animals at dose levels of 100, 250, 500 or 1,000 mg/kg bw/day after start of treatment (Day 6) until day of death/sacrifice of all animals (Day 22, Day 23, Day 16 and Day 15, respectively).

At 50 mg/kg bw/day, mean total food consumption was decreased at the end of the study compared to the food consumption of the 25 mg/kg bw/day dose group animals. Slightly reduced food consumption was also noted in the low dose (25 mg/kg bw/day) animals during the treatment period compared to pre-treatment values.

Table 5.6.2- 16: Mean food consumption during gestation#

	Dose level [mg/kg bw/day]					
	25	50	100	250	500	1,000
Mean Food Consumption [g/animal/day]						
Day 0-3	111.8	81.3	98.8	38.4	81.3	74.8
Day 3-6	107.3	89.8	100.9	78.5	62.7	86.4
Day 6-8	97.5	93.8	80.0	90.3	34.4	32.1
Day 8-10	85.7	88.5	72.6	71.8	25.8	17.3
Day 10-13	93.1	89.3	33.6	25.9	7.5	2.2
Day 13-16	70.2	72.1	1.8	2.6	1.3	-
Day 16-19	96.7	68.3	1.9	3.0	-	-
Day 19-23	84.9	55.9	-	-	-	-
Day 23-26	81.4	63.0	-	-	-	-
Day 26-29	84.0	23.5	-	-	-	-
Total Day 0-29	2569.0	2050.3	-	-	-	-

Non-pregant dams were excluded from the means
- No data available due to mortality rate of 100 % of pregnant dams in the respective groups

3. Necropsy findings:

Necropsy findings in the animals found dead, killed moribund or killed after abortion consisted of beige discoloured heart, liver and kidney. In most animals the stomach showed petechial bleedings and in some was filled with feed mash. No macroscopically visible changes were observed at necropsy of the animals from the 25 and 50 mg/kg bw/day group.

4. Caesarean section data:

One animal at 25 mg/kg bw/day, 50 mg/kg bw/day and 250 mg/kg bw/d were not pregnant. The animal of the 50 mg/kg bw/day group which aborted had six dead foetuses. No abnormalities were observed at caesarean section in the remaining animals from these groups.

Table 5.6.2- 17: Results of gestation and Caesarean section

Parameter	Dose level [mg/kg bw/day]	
	25	50
No. pregnant / no. mated	3 / 4	3 / 4
No of abortions	0	1
No. dams with live foetuses	3	2
Mean no. corpora lutea/dam	8.7	8.5
Mean no. implantation sites/dam	8.3	8.0
Pre-implantation loss (% of corpora lutea)	4.2	6.3
Post-implantation loss (% implants)	0.0	5.6
Mean no. of resorptions/dam	0.0	0.0
Early resorptions/dam	0.0	0.0
Dead foetuses/dam	0.0	0.0

5. Foetal data

Foetal parameters were not affected by treatment with fluopicolide neither at 25 mg/kg bw/day nor at 50 mg/kg bw/day.

Table 5.6.2- 18: Foetal data

Parameter	Dose level [mg/kg bw/day]	
	25	50
Total no. of live foetuses	25	15
Mean no. live foetuses/dam	8.3	7.5
Sex ratio (% males)	Not determined	
Mean foetal weight (g)	37.8	35.3
Crown-rump length (mm)	89.6	89.6
Mean placental weight (g)	4.6	4.4

III. Conclusions

Based on the results of this study, a dose level around 50 mg/kg bw/day was considered to be an appropriate high dose for the main study.

Assessment and conclusion by applicant:

The study is valid and acceptable to aid in the selection of doses for the main developmental study in rabbits.

Data Point:	KCA 5.6.2/04
Report Author:	[redacted]
Report Year:	2004
Report Title:	AE C638206 Code: AE C638206 00 1C99 0005 Rabbit Oral Developmental Toxicity (Teratogenicity) Study
Report No:	C044368
Document No:	M-202513-02-1
Guideline(s) followed in study:	EU (=EEC): 88/302; JMAF: 59 NohSan 4200; OECD: 414; USEPA (=EPA): OPPTS 870.3700
Deviations from current test guideline:	Method: Deviations from current guideline SANCO/3029/99 rev. 4: There is no specificity or linearity data presented and the accuracy and precision data are determined from the test suspension samples in the toxicological study. However, there are 6 samples per concentration level with mean accuracies between 70-110% and RSD > 20%. Considering that this analytical method is validated in support of toxicological cold studies, the method validation is considered fit for purpose.; Study: The following deviations from the OECD-Guideline 414 (2018) occurred: according to the guideline, the highest dose should be chosen with the aim to induce some developmental and/or maternal toxicity (clinical signs or a decrease in body weight) but not death (exceeding approx. 10%) or severe suffering. In the present study the high dose resulted in a mortality rate > 10%. Therefore, the number of females with implantation sites that were available at necropsy (fewer than 16 animals) limits the validity of the assessment of the high dose results. Although this outcome might suggest that a new study is required to allow examination of foetuses from dams experiencing tolerable maternal toxicity, the small dose space between 20 mg/kg bw/day (NOEL) and 60 mg/kg bw/day makes the selection of a dose that might be tolerated but induce some toxicity difficult. Therefore, the study is considered to be sufficient to assess the developmental toxicity of fluopicolide.
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Data Point:	KCA 5.6.2/06
Report Author:	[redacted]
Report Year:	2003
Report Title:	The Himalayan rabbit (Oryctolagus cuniculus L.): Spontaneous incidences of endpoints from prenatal developmental toxicity studies
Report No:	M-619222-01-1
Document No:	M-619222-01-1
Guideline(s) followed in study:	--
Deviations from current test guideline:	--
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	not applicable
Acceptability/Reliability:	Yes

Executive Summary:

The present study was conducted in order to determine the effects of fluopicolide on maternal health and embryonic and foetal development. Groups of 23 mated female Himalayan rabbits received fluopicolide by oral gavage in 1% methylcellulose once daily at the dose levels of 0, 5, 20 or 60 mg/kg bw from Day 6 to day 28 of gestation (Day 0: day of mating) and were sacrificed on Day 29 of gestation. The dosing volume was 5 mL/kg bw.

Behaviour and state of health were observed daily in all groups. Body weight and food consumption were determined regularly throughout the study.

At necropsy the dams were examined for macroscopically visible changes. Gravid uterus weight was recorded. The uterus was opened and the number of live and dead foetuses and the number of conceptuses undergoing resorption were determined. Body weights, crown-rump lengths, sex ratios of the foetuses and placental weights were determined. The foetuses were examined for external, visceral and skeletal anomalies.

Three animals of the 60 mg/kg bw/day dose group were found dead and 15 animals of this group were killed after premature delivery during Day 22-29 of gestation. These animals showed decreased defecation, reduced hay consumption, hypoactivity, bristling coat, pultaceous faeces, and discoloured urine. One animal of the 20 mg/kg bw/day dose group was killed after premature delivery on Day 28 of gestation. This animal also showed decreased defecation and reduced hay consumption. Since up to 20% abortions are covered by historical control data this isolated single premature delivery is considered incidental and not treatment-related.

Body weight gains and food consumption were markedly decreased in animals of the 60 mg/kg bw/day dose group throughout the treatment period (-86%). In addition, gravid uterus weights were slightly lower at the same dose level.

At necropsy, tautly filled stomach, red liquid in urinary bladder and uterus as well as yellowish discolouration of the liver were observed in single animals of the high dose group. No compound-related effects were observed in the low and intermediate dose group.

Dead foetuses were present in most premature deliveries. Mean foetal body weights and crown-rump lengths were decreased in foetuses at 60 mg/kg bw/day. Litter size, number of live and dead foetuses as well as sex ratios remained unaffected by the administration of the test compound. Likewise, incidences of early and late conceptuses undergoing resorption were not affected by the administration of the test substance. External, skeletal and visceral examination of the foetuses did not reveal any compound-related effects.

In conclusion, oral administration of fluopicolide to pregnant rabbits at the dose of 60 mg/kg bw/day caused increased incidences of premature deliveries, reduced foetal crown-rump lengths and foetal weights. All these findings are considered secondary to severe maternal toxicity as evidenced by mortality and decreases in body weight gain due to drastically reduced feed consumption at the highest tested dose.

At doses up to and including 20 mg/kg bw/day, fluopicolide did not cause any maternal toxicity or embryotoxicity. There was no evidence of treatment-related teratogenic effects at any dose level.

Therefore, the No Observed Adverse Effect Level (NOAEL) of the present study is 20 mg/kg bw/day for maternal toxicity and for developmental toxicity.

I. Materials and Methods

A. Materials

1. Test material:

Identification:	AE C638206
Batch:	PP/241024/2 & PP241067/1
Purity:	97.8% w/w (PP/241024/2 & PP241067/1)

2. Vehicle and/or positive control:

1 % w/v methyl cellulose in deionised water

3. Test animals

Species:	Rabbit
Strain:	Chbb:HM(SPF) Himalayan rabbit
Age:	approximately 5-10 months
Weight at start:	2145-3095 g
Source:	[redacted]
Acclimation phase:	yes
Diet:	commercial diet for laboratory rabbits Ssniff K-H (V2333)1 ad libitum in food racks, additionally 40-50 g hay daily
Water:	tape water, ad libitum from automatic dispensers
Housing:	in fully air-conditioned rooms in V2A-steel-cage type: HD3 Fa. Hulskamp
Temperature:	approx. 22 ± 3 °C
Humidity:	approx. 50 ± 20%
Air changes:	16-20 air changes/hour
Photoperiod:	12 hours

B. Study design

1. In-life dates: February 28 to November 22, 2000

2. Animal assignment and treatment:

The test animals were assigned randomly (computer-generated algorithm) to the following groups (see Table 5.6.2- 19).

Table 5.6.2- 19: Study design

Test group	Dose level (ppm)	Number of females
1	0	23
2	5	23
3	20	23
4	60	23

3. Duration of dosing:

Female animals showing sperm in the vaginal smear after mating received fluopicolide orally by gavage once daily from Day 6-28 of gestation.

4. Dose preparation and administration:

Female animals showing sperm in the vaginal smear after mating received fluopicolide at the dose levels of 0, 5, 20 or 60 mg/kg bw/day as an aqueous preparation in 1% methyl cellulose orally by gavage once daily from Day 6-28 of gestation. All groups received a dose volume of 5 mL/kg bw, with adjustment of the individual volume to the most recently recorded body weight. The test suspension was prepared daily, immediately before dosing.

5. Mating:

Virgin female animals were mated with sexually mature males in the ratio 1 male : 1 female and were caged individually after the detection of sperm in vaginal smears. The day of mating is defined as Day 0 of gestation. Pregnancy was confirmed at necropsy by the detection of implantation sites or normally developed corpora lutea.

6. Statistics:

The statistical evaluation is based on the assumption of a monotone dose-response relationship. Statistical comparisons of the low dose groups with the simultaneous control group were only carried out if significant effects were detectable in the high dose group. In the univariate analysis, two-sided questions (body weight of dams, relative food consumption, crown-rump length, foetal weight and placental weight) were generally tested as follows: a two-sided comparison with the high dose group was followed by a one-sided test for the low-dose group.

In case of the caesarean section data of the foetuses (crown-rump length, foetal weight and placental weight), multivariate statistics were first of all calculated and used in selecting relevant dose groups. For the individual parameters, sequential comparisons with the high dose group and sequential tests at the 5% level for the low dose were then conducted.

The t-tests and the test statistics of Wilks are based on common variance estimations for all study groups. For the Wilcoxon test the exact distribution of the mean ranks was calculated.

In the case of the daily food consumption of the dams, the mean consumption per 100 g body weight was always calculated between two successive measurement times and evaluated by the rank sum test after Wilcoxon. In examining the body weights of the dams, the change in weight was determined in comparison to the initial weight. The univariate evaluation was carried out using t-tests.

The caesarean section data of the foetuses were used to calculate litter mean values. Multivariate evaluation was carried out using the test statistics of Wilks. In the univariate analysis, t-tests were used.

The number of corpora lutea, implantation sites and live foetuses, and dead embryonic primordia undergoing resorption in the animals were likewise analysed using one sided Wilcoxon tests.

The findings obtained at autopsy and at organ cross-section and skeletal examination of the foetuses were evaluated separately for the foetuses and for the litters by Jack-knife t-test at a significance levels of 5%. It was examined whether the relative frequencies of findings in the dose groups deviated from those findings in the control group.

C. Methods

1. Observations:

All animals were examined before the start of the study and were shown to be in good general health condition. The behaviour and general health condition of the animals were observed several times daily.

2. Body weights and food consumption:

Body weights were recorded on Days 0, 3, 6, 8, 10, 13, 16, 19, 23, 26 and 29 of gestation, and food consumption was determined between Days 0-3, 3-6, 6-8, 8-10, 10-13, 13-16, 16-19, 19-23, 23-26 and 26-29.

3. Caesarean section:

The animals were sacrificed on Day 29 of gestation by intravenous injection of 1.61 HOECHST® and the foetuses removed by Caesarean section. Gravid uterus weight was determined. All animals were autopsied and checked for macroscopically visible changes, with emphasis on the uterus. The live and dead foetuses in the uterus as well as the conceptuses undergoing resorption and corpora lutea were counted and examined macroscopically.

4. Foetal evaluation:

The foetuses, the placentae and conceptuses undergoing resorption were removed from the uterus, weighed or measured and examined for gross external abnormalities. Afterwards the foetuses were killed by CO_2-asphyxia and the crown-rump length recorded. All foetuses were fixed in alcohol, necropsied, sexed and checked for anomalies of the internal organs. Eyes, brain, heart and kidneys were cross-sectioned and examined for anomalies. All carcasses were placed in a solution of potassium hydroxide for clearing and stained with alizarin red S and Alcian blue. The skeletons (bone and cartilage) were examined and checked for stage of development and abnormalities with the aid of a magnifier. The foetuses found dead at caesarean section were examined for external anomalies.

Visceral and skeletal changes were subdivided into four categories (major defects, minor defects, variations and retardations) based on the severity and/or the spontaneous incidence of the finding.

II. Results and Discussion

A. Observations:

1. Maternal data:

Three animals of the high dose group were found dead and 15 animals of this group were killed after premature delivery from Day 22-29 of gestation. These animals showed decreased defecation, reduced hay consumption, hypoactivity, bristling coat, pultaceous faeces and discoloured urine. One animal of this dose group showed increased salivation. One animal from the intermediate dose group was killed after premature delivery on Day 28 of gestation. This animal also showed decreased defecation and hay consumption. Since up to 20% abortions are covered by historical control data this single isolated premature delivery is considered incidental and not treatment-related.

Table 5.6.2- 20: Maternal mortality data until study termination

	Dose level [mg/kg bw/day]			
	0	**5**	**20**	**60**
Total animal number per group	23	23	23	23
Maternal mortality				
No. of animals **found dead**	0	0	0	3
Day(s) of death/sacrifice	-	-	-	24-25, 29
No. of animals **killed moribund**	0	0	0	0
Day(s) of death/sacrifice				
No. of animals **killed after premature delivery**	0	0	1	15
Day(s) of death/sacrifice			28	22-28
Total number animals dead/sacrificed before study termination	0	0	1	18

2. Body weight and food consumption:

Body weight gains were markedly decreased in the animals from the high dose group throughout the treatment period (Day 6-29: -86%). Body weights were statistically significant lower than control on Days 26 and 29 of gestation.

In addition, gravid uterus weights were slightly lower at 60 mg/kg bw/day compared to control.

An overview is given in Table 5.6.2-21.

Table 5.6.2- 21: Mean maternal body weight and body weight gain

	Dose level [mg/kg bw/day]					
	0	**5**		**20**	**60**	
Body weight [g] *(% difference to control)*						
Day 0	2491.9	2424.5	*(-3)*	2503.6 *(±0)*	2496.6	*(±0)*
Day 3	2556.5	2528.0	*(-1)*	2590.8 *(+1)*	2598.0	*(+2)*
Day 6	2580.9	2531.7	*(-2)*	2599.6 *(+1)*	2614.2	*(+1)*
Day 8	2571.8	2513.9	*(-2)*	2587.6 *(+1)*	2607.8	*(+1)*
Day 10	2570.1	2519.4	*(-2)*	2603.0 *(+1)*	2595.6	*(+1)*
Day 13	2596.9	2537.5	*(-2)*	2616.6 *(+1)*	2614.2	*(+1)*
Day 16	2653.6	2601.8	*(-2)*	2676.6 *(+1)*	2640.8	*(±0)*
Day 19	2677.8	2624.0	*(-1)*	2683.8 *(+1)*	2660.2	*(±0)*
Day 23	2729.0	2656.0	*(-3)*	2728.3 *(±0)*	2693.8	*(-1)*
Day 26	2758.9	2725.5	*(-2)*	2798.4 *(±0)*	2668.6*	*(-4)*
Day 29	2852.6	2785.2	*(-2)*	2859.8 *(±0)*	2652.4*	*(-7)*
Day 29*	2476.9	2424.4	*(-2)*	2480.0 *(±0)*	2348.5	*(-5)*

	Dose level [mg/kg bw/day]					
	0	5		20		60	
Cumulative body weight gain [g] *(% difference to control)* [#]						
Day 0-6	89.0	107.2	*(+20)*	96.0	*(+8)*	117.6	*(+32)*
Day 6-29 [b]	271.7	253.5	*(-7)*	260.2	*(-4)*	38.2	*(-86)*
Day 0-29	360.7	360.7	*(±0)*	356.2	*(-1)*	155.8	*(-57)*
Gravid uterus weight [g] *(% difference to control)*						
Day 29	375.7	360.8	*(-4)*	379.8	*(+1)*	303.9	*(-19)*

* Statistically different from control, $p \leq 0.05$
[#] No statistical analyses were performed.
[a] Corrected for gravid uterus weight
[b] Treatment period only
Animals not surviving to day 29 after mating, with total litter loss or non pregnant animals are excluded from the means

Likewise, food consumption was markedly decreased in the animals from the high dose group throughout the treatment period (especially in second half of gestation).

Table 5.6.2- 22: Mean food consumption during gestation

	Dose level [mg/kg bw/day]					
	0	5		20		60	
Mean Food Consumption [g/100 g body weight] *(% difference to control)*						
Day 0-3	3.5	3.7	*(+6)*	3.4	*(-3)*	4.3	*(+23)*
Day 3-6	4.1	4.1	*(±0)*	4.1	*(±0)*	4.7	*(+15)*
Day 6-8	3.5	3.5	*(±0)*	3.4	*(-3)*	3.5	*(±0)*
Day 8-10	3.6	3.4	*(-6)*	3.4	*(-6)*	3.2	*(-11)*
Day 10-13	3.6	3.4	*(-6)*	3.4	*(-6)*	3.2	*(-11)*
Day 13-16	3.4	3.3	*(-3)*	3.1	*(-9)*	2.8	*(-18)*
Day 16-19	3.7	3.6	*(-3)*	3.4	*(-8)*	2.7	*(-27)*
Day 19-23	3.7	3.6	*(-3)*	3.5	*(-5)*	3.0	*(-19)*
Day 23-26	3.5	3.7	*(+6)*	3.5	*(±0)*	2.0*	*(-43)*
Day 26-29	3.5	3.6	*(+3)*	3.4	*(-3)*	1.6*	*(-54)*

* Statistically different from control, $p \leq 0.05$
Animals not surviving to day 29 after mating or resorptions or non pregnant animals are excluded from the means

Body weight, body weight gains and food consumption remained generally unaffected by the administration of fluopicolide at 20 mg/kg bw/day and below. However, the one dam at the mid dose level that had a premature delivery on Day 28 (No. 142) also showed markedly reduced feed consumption during the three days prior death (19% of control; Figure 5.6.2- 3).

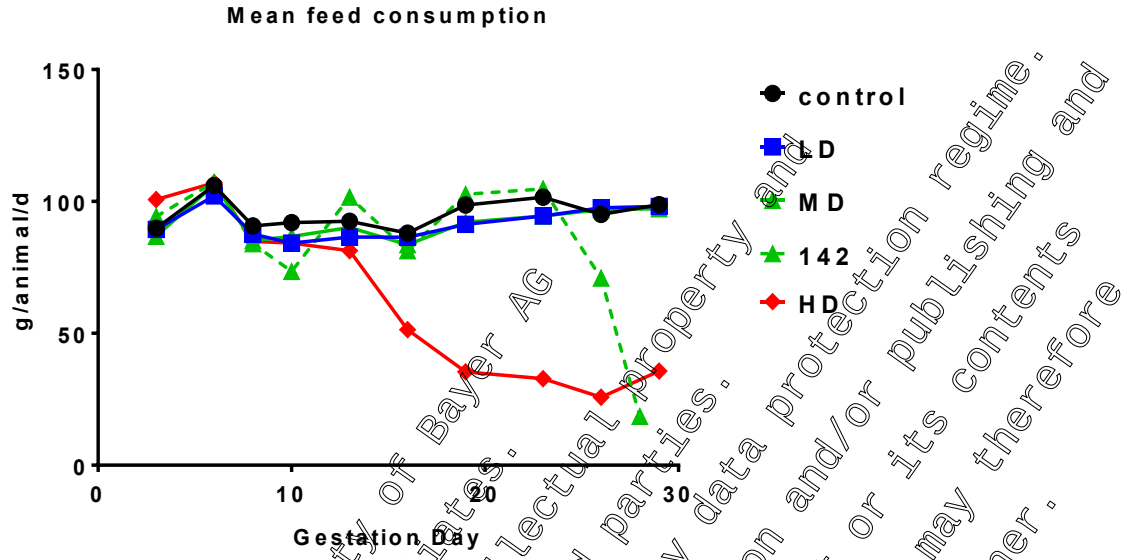


Figure 5.6.2- 3: **Mean food consumption during treatment period in g/animal/day. The food consumption of the one dam at the mid dose level that had a premature delivery on Day 28 (No. 142) is shown separately as green dashed line. LD = low dose (5 mg/kg bw/day) MD = mid dose (20 mg/kg bw/day) HD = high dose (60 mg/kg bw/day)**

3. Necropsy findings:

Gross examination of dams at necropsy showed tautly filled stomach, red liquid in urinary bladder and uterus as well as yellowish discolouration of the liver in single animals of the high dose group (Table 5.6.2- 23).

No treatment-related effects were observed in the animals from the low and intermediate dose group.

Table 5.6.2- 23: **Gross findings at necropsy** (Number of animals affected)

Parameter	**Dose level [mg/kg bw/day]**			
	0	**5**	**20**	**60**
Number of animals examined	23	23	23	23
Stomach				
Tautly filled (full of mash)	0	0	0	6
Urinary bladder				
Filled with red fluid	0	0	0	2
Uterus				
Filled with red fluid	0	0	0	1
Liver				
Lobus dexter access. yellow discoloured	0	0	0	1

4. Caesarean section data:

With the exception of three females at 5 mg/kg bw/day and one female at 20 mg/kg bw/day, all animals became pregnant. In addition, one female of the control group had total pre-implantation loss.

In most cases in which premature delivery occurred, dead foetuses were observed. At study termination, one dead foetus occurred in the control, low and high dose group each, and three dead foetuses were observed in the intermediate dose group. These incidences of dead foetuses are not unusual in the rabbit strain used and therefore, a compound-related effect is not evident.

An overview including all other Caesarean section parameters is given in the following tables.

Table 5.6.2- 24: Results of gestation and Caesarean section

Parameter	Dose level [mg/kg bw/day]				
	0	5	20	60	HCD#
No. pregnant / no. mated	23 / 23	20 / 23	22 / 23	23 / 23	
No. of intercurrent death	0	0	0	3	
No. dams with premature delivery	0 (0.0%)	0 (0.0%)	1 (4.5%)	15 (65.2%)	0.0%-20.0%
No. dams with live foetuses	22[a]	20	21	5	
Mean no. corpora lutea/dam	8.3	8.3	8.4	8.2	6.4-10.0
Mean no. implantation sites/dam	7.3	7.6	7.8	7.0	5.1-8.5
Pre-implantation loss (% of corpora lutea)	10.8	9.2	7.7	16.1	
Post-implantation loss (% implants)	4.1	12.2	10.9	10.3	
Total intrauterine deaths/dam	0.32	0.95	0.76	0.60	0.0-1.25
Early resorptions/dam	0.27	0.90	0.62	0.40	
Dead foetuses/dam	0.05	0.05	0.14	0.20	

[a] One pregnant female had total pre-implantation loss

[#] Ranges of studies performed with animals of the same strain and breeder between 1968 and 1999 including 1144 litters (Viertel B, Trieb G. The Himalayan rabbit (Oryctolagus cuniculus L.): spontaneous incidences of endpoints from prenatal developmental toxicity studies. Lab Anim. 2003 Jan;37(1):19-36; M-619222-01-1)

5. Foetal Data:

Mean foetal body weights and crown-rump lengths were statistically significantly decreased in foetuses at 60 mg/kg bw/day. Mean number of live foetuses (per dam) remained unaffected by the administration of the test substance. Likewise, sex ratio of the foetuses was not altered by the administration of the test substance.

Table 5.6.2- 25: Foetal data

Parameter	Dose level [mg/kg bw/day]			
	0	5	20	60
Total no. of live foetuses	157	132	147	32
Mean no. live foetuses/dam	7.1	6.6	7.0	6.4
Sex ratio (% males)	53.5	58.3	51.7	43.8
Mean foetal weight (g)	37.8	38.8	39.4	32.4*
Crown-rump length (mm)	93.3	93.9	95.1	88.1*
Mean placental weight (g)	4.91	5.13	5.00	4.53

* Statistically different from control, $p \leq 0.05$

The increased incidence of premature deliveries with mainly dead foetuses and the reduced foetal weights and crown-rump lengths in the highest dose group in the present study are considered as secondary consequences of severe maternal toxicity as evidenced by mortality and decreases in body weight gain due to markedly reduced feed consumption.

As shown in Table 5.6.2- 26, mean food consumption was drastically reduced to 9% of control in the high dose dams that died or had premature deliveries from gestation Day 19 until death or sacrifice whereas the high dose dams that survived until termination showed only a food consumption reduction to 60% of control which is in good agreement with published results in rabbits (Matsuzawa *et al*. 1981[30]; Petrere *et al*. 1993[31]; Cappon *et al*. 2005[32]; Menchetti *et al*. 2015[33]).

[30] Matsuzawa T, Nakata M, Goto I, Tsushima M. Dietary deprivation induces foetal loss and abortion in rabbits. Toxicology. 1981;22(3):255-9.

[31] Petrere JA, Rohn WR, Grantham LE 2nd, Anderson JA. Food restriction during organogenesis in rabbits: effects on reproduction and the offspring. Fundam Appl Toxicol. 1993 Nov;21(4):517-22.

[32] Cappon GD, Fleeman TL, Chapin RE, Hurtt ME. Effects of feed restriction during organogenesis on embryo-foetal development in rabbit. Birth Defects Res B Dev Reprod Toxicol. 2005 Oct;74(5):424-30.

[33] Menchetti L, Brecchia G, Canali C, Cardinali R, Polisca A, Zerani M, Boiti C. Food restriction during pregnancy in rabbits: effects on hormones and metabolites involved in energy homeostasis and metabolic programming. Res Vet Sci. 2015 Feb;98:7-12

Table 5.6.2- 26: Mean maternal food consumption in g/animal/day during gestation *(% of control)*

Parameter	Dose level [mg/kg bw/day]				
	0	5	20	60	
				Died or aborted during gestation	Survived until termination
Number of dams	21	20	21	18	5
Day 6-19	92.4	87.2 *(94%)*	87.5 *(95%)*	64.0 *(69%)*	79.9 *(86%)*
Day 19- sacrifice[a]	98.5	96.7 *(98%)*	70.0 *(71%)*	9.1 *(9%)*	58.9 *(60%)*

[a] death in case of 3 animals in the high dose group

External, skeletal and visceral examination:

External, skeletal and visceral examination of live foetuses did not reveal any treatment-related abnormalities. Minor defects and variations observed were within the historical range of the rabbit strain used, without any dose-dependency and/or statistical evaluation did not reveal differences between the groups.

Furthermore, dead foetuses, foetuses of dams which died or which had premature deliveries did not show any major defects.

III. Conclusions

In conclusion, oral administration of fluopicolide to pregnant rabbits at the dose of 60 mg/kg bw/day caused increased incidences of premature deliveries, reduced foetal crown-rump lengths and reduced foetal and placental weights. All these findings are considered secondary to severe maternal toxicity as evidenced by mortality and decreases in body weight gain due to drastically reduced feed consumption at the highest tested dose. External, skeletal and visceral examination of live foetuses did not reveal any treatment-related abnormalities.

At doses up to and including 20 mg/kg bw/day, fluopicolide did not cause any maternal toxicity or embryotoxicity. There was no evidence of treatment-related teratogenic effects at any dose level.

Therefore, the No Observed Adverse Effect Level (NOAEL) of the present study is 20 mg/kg bw/day for maternal toxicity and for developmental toxicity.

Assessment and conclusion by applicant:

The study was conducted according to OECD 414 and is valid and acceptable to assess the developmental toxicity potential of fluopicolide. A NOAEL of 20 mg/kg bw/d was determined from this study for maternal and developmental toxicity.

Data Point:	KCA 5.6.2/07
Report Author:	[redacted]
Report Year:	2018
Report Title:	Fluopicolide: Review of potential for classification for reproductive and developmental toxicity
Report No:	M-638869-01-1
Document No:	M-638869-01-1
Guideline(s) followed in study:	None
Deviations from current test guideline:	not specified
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	not applicable
Acceptability/Reliability:	Yes

Executive Summary

Fluopicolide is due for review with respect to the potential for classification and labelling (Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures). This review has been undertaken to assess the potential for classification of fluopicolide as a reproductive toxicant.

On the basis of the results of a two-generation reproduction study in the rat and prenatal developmental toxicity studies in rats and rabbits, fluopicolide should not be classified as reproductive toxicant based on the ECHA Guidance on the application of the CLP criteria.

I. Reproductive Toxicity

A two-generation study has been conducted in the rat ([redacted] 2003; M-232532-01-1) which is consistent with current OECD test guideline 416. The F0 generation, which comprised 28 males and 28 females in each group, received diet with inclusion levels of 0, 100, 500 or 2,000 ppm for 10 weeks before pairing and throughout mating, gestation, littering and lactation. Offspring survival and growth to weaning were evaluated at which point 24 male and 24 female offspring per group were selected to form the F1 generation. Both sexes received diets as did their parents for a minimum of 10 weeks from selection, throughout pairing, gestation, littering and lactation. Sexual maturation, fertility and reproductive capacity of the F1 generation were assessed and resulting F2 offspring were monitored for survival and development until weaning.

Parental toxicity was achieved at the highest dose tested but fertility and reproductive performance were unaffected in both generations. The only effect in pups was a lower body weight at the highest dose which attained statistical significance at Day 14, for male and female pups from both generations, coinciding with the time when the offspring start to eat the diet, suggesting a palatability effect and/or systemic toxicity and not a developmental effect. Furthermore, no adverse effect on lactation was indicated.

The study design included endocrine sensitive endpoints including oestrus cyclicity, parturition, sexual maturation and these were not affected by treatment with fluopicolide.

Macroscopic examination at necropsy of the unselected F1 offspring or the F2 offspring did not indicate any adverse effect of treatment. Differences in spleen and thymus weights of the offspring treated at 2,000 ppm were generally attributed to the lower body weights in this group, although body weight-relative spleen weights were low for female offspring at this dose for both generations.

Macroscopic examination at necropsy of the F0 and F1 adults revealed no treatment-related findings. At 2,000 ppm, kidney and liver weights were high for males and females in both generations, when compared with the controls. Body weight-relative liver weights were also slightly high for the F1 females treated at 500 ppm (considered likely to be an adaptive liver change). Treatment-related histopathological change was observed in the liver and kidneys of males and females at 2,000 ppm and in the liver of males at 500 ppm.

Absolute and body weight-relative spleen weights were slightly low for the F0 parental animals treated at 2,000 ppm and absolute spleen weights were also low for the F1 females in this group. Histopathological examination of the spleen was not conducted, and the significance of the weight changes cannot be determined. Sperm analysis in males (control and high dose groups only) of both generations did not reveal any treatment-related findings.

The dietary concentration of 500 ppm should be considered as the NOAEL based on the likelihood of the increased liver weights in F1 females being an adaptive change and not an adverse toxicological effect. The minimum mean achieved dosages for the F0 animals at this NOAEL (500 ppm) are 25.5 mg/kg bw/day for the males and 32.9 mg/kg bw/day for the females. The NOEL for developing offspring is considered to be 500 ppm, equivalent to a minimum mean achieved dosage of 39.8 mg/kg bw/day for F0 females during gestation and lactation. The NOEL for reproductive parameters is considered to be 2,000 ppm, equivalent to a mean achieved dosage of at least 103.4 mg/kg bw/day for F0 males and at least 127.3 mg/kg bw/day for F0 females before pairing.

With reference to the ECHA Guidance on the application of the CLP criteria, there are no results from this study of reproduction and fertility in the rat that determine a need for classification, i.e. there are no adverse effects sexual function and fertility and no adverse effect on prenatal or postnatal development of the offspring, in the presence of parental toxicity. Lower body weight from postnatal Day 14, in the F1 offspring at 2,000 ppm, was not a developmental effect but a palatability effect and/or systemic toxicity resulting from direct consumption of the diet. Furthermore, no adverse effect on lactation was indicated.

II. Developmental Toxicity

1. Rat

A study was conducted in order to determine the effects of fluopicolide on the maternal state of health, embryonic and foetal development when administered orally during pregnancy ([redacted] 2004; M-202155-02-1). For the definitive study, groups of 25 mated female Sprague Dawley rats received fluopicolide by gavage in 1% methylcellulose at dose levels of 5, 60 or 700 mg/kg bw/day on Days 7-20 of pregnancy (Day 0: day of mating, Day 1: day of sperm detection) and were terminated on Day 21 of pregnancy. The study design was based on international test guidelines (including OECD TG 414 adopted 1981). Although the study predates the current version of the guideline (adopted in 2018), there are no significant deviations other than the omission of the recently included thyroid hormone measurements, thyroid weight and histopathology and foetal anogenital distance; the treatment period is consistent with the current guideline requirement. However, with respect to dose selection, the current test guideline describes two- to four-fold intervals between doses as being frequently optimal for setting the descending dose levels and that the addition of a fourth test group is often preferable to using very large intervals (e.g. more than a factor of 10) between dosages. At the time of the study conduct, the test guideline required only that the intermediate dose be located geometrically between the low and high dose levels and the doses were selected based on a factor of 12 and on the results of a preliminary study ([redacted] 2000; M-198488-01-1).

The NOAEL for maternal toxicity and fetotoxicity was 60 mg/kg bw/day, based on decreased body weight gain in dams (8% of controls for Days 7-21) and a reduction in mean foetal body weight (8%) and crown–rump length (4%) in foetuses at 700 mg/kg bw/day. Litter size, number of live and dead foetuses as well as sex ratios remained unaffected by the administration of the test compound. Likewise, incidences of early and late conceptuses undergoing resorption were not affected by the administration of the test compound.

Visceral and skeletal findings were subdivided into four categories (major defects, minor defects, variations, and retardations) based on the severity of the finding and/or the spontaneous incidence of the finding. OECD TG 414 (2018) defines malformation/major abnormality as structural change considered detrimental to the animal (may also be lethal) and usually rare and, variation/minor abnormality as structural change considered to have little or no detrimental effect on the animal; may be transient and may occur relatively frequently in the control population. Fluopicolide was found not to be teratogenic in the rat; the incidence of major malformations was low and clearly incidental to the administration of fluopicolide.

The incidence of foetuses/litters with specific minor external/visceral defects or variations was low and not statistically significant (see Table 5.6.2- 27); there was no evidence of any treatment-related increase in incidence or dose-related trend. The findings relating to the presence of blood or haematoma are due to handling at necropsy. All findings were considered incidental to treatment.

Table 5.6.2- 27: Incidence of external/visceral variations and minor defects including those detected at body cross-section# (no. (%) of foetuses and litters)

Variation / Minor Defect	Dose Level (mg/kg bw/day)			
	0	5	60	700
Number of foetuses examined	148/136[#] (284)	150/141[#] (291)	153/144[#] (297)	142/132[#] (274)
Number of litters examined	22	21	22	21
Thoracic cavity - blood	1 (0.7) 1 (4.5)	0 (0.0) 0 (0.0)	0 (0.0) 0 (0.0)	1 (0.7) 1 (4.8)
Thoracic cavity – blood[#]	1 (0.7) 1 (4.5)	0 (0.0) 0 (0.0)	0 (0.0) 0 (0.0)	0 (0.0) 0 (0.0)
Heart pericardium - blood	1 (0.7) 1 (4.5)	1 (0.7) 1 (4.8)	0 (0.0) 0 (0.0)	0 (0.0) 0 (0.0)
Abdominal cavity – blood[#]	2 (1.5) 2 (9.1)	5 (3.5) 5 (23.8)	4 (2.8) 3 (13.6)	3 (2.3) 2 (9.5)
Lung lobus inferior medialis – reduced in size	0 (0.0) 0 (0.0)	1 (0.7) 1 (4.8)	0 (0.0) 0 (0.0)	0 (0.0) 0 (0.0)
Liver lobes -haematoma[#]	1 (0.7) 1 (4.5)	2 (1.4) 2 (9.5)	1 (0.7) 1 (4.5)	4 (3.0) 4 (19.0)
Kidney displaced mediad, right	0 (0.0) 0 (0.0)	0 (0.0) 0 (0.0)	1 (0.7) 1 (4.5)	0 (0.0) 0 (0.0)
Kidney displaced caudad – right[#]	0 (0.0) 0 (0.0)	0 (0.0) 0 (0.0)	0 (0.0) 0 (0.0)	1 (0.8) 1 (4.8)
Kidney pelvis distended – unilateral	5 (3.4) 3 (13.6)	1 (0.7) 1 (4.8)	2 (1.3) 2 (9.1)	5 (3.5) 3 (14.3)
Kidney pelvis distended – unilateral or bilateral[#]	1 (0.7) 1 (4.5)	1 (0.7) 1 (4.8)	3 (2.1) 3 (13.6)	5 (3.8) 4 (19.0)
Kidney pelvis distended – overall incidence	6 (2.1) 4 (18.2)	2 (0.7) 2 (9.5)	5 (1.7) 4 (18.2)	10 (3.6) 4 (19.0)

Variation / Minor Defect	Dose Level (mg/kg bw/day)			
	0	5	60	700
Ureter distended – unilateral or bilateral[#]	1 (0.7) 1 (4.5)	0 (0.0) 0 (0.0)	0 (0.0) 0 (0.0)	4 (3.0) 3 (14.3)
Ureter distended – overall incidence	1 (0.4) 1 (4.5)	0 (0.0) 0 (0.0)	0 (0.0) 0 (0.0)	4 (1.5) 3 (14.3)

Overall incidence includes all foetuses examined irrespective of examination technique

Foetuses from the high dose group showed increased incidences of a small number of minor skeletal defects and evidence of retarded development. To better understand the nature of the observed minor defects, it is necessary to consider the individual foetal data. This enables collation of the findings for each foetus and aids visualisation of the anomalies. The selected minor defects are common to several foetuses and several litters in the high dose group, as shown below:

0 mg/kg bw/day: **Litter 14 Foetus R07** RIB, 10th, Right, Wavy

5 mg/kg bw/day: **Litter 43 Foetus L06** RIB, 6th-8th, Right, Thickened, RIB, 10th-11th, Right, Wavy

60 mg/kg bw/day: **Litter 60 Foetus L05** (2.7 g body weight - litter mean 3.785g)

THORACIC VERT.ARCH, 9th, Right, Fused with attached rib, THORACIC VERT.ARCH, 9th, Bilateral, Dysplasia, THORACIC VERT.ARCH, 10th-13th, Bilateral, Aplasia, THORACIC VERT.CENTRA, 8th, Dysplasia,

THORACIC VERT.CENTRA, 8th, Dislocated, THORACIC VERT.CENTRA, 9th-13th, Aplasia,

LUMBAR, SACRAL, CAUDAL VERTEBRA, All vertebrae, Aplasia, Major defect

RIB, Bilateral, Anlage of only 9, RIB, 9th, Left, Dysplasia

PELVIC GIRDLE, Ilium & Ischium, Bilateral, Dysplasia Major defect

700 mg/kg bw/day:

Litter 71 Foetus R07 THORACIC VERT.CENTRA, 11th, Fragmented

Litter 74 Foetus R03 THORACIC VERT.CENTRA, 11th & 12th, Fragmented

Litter 75 Foetus L05 RIB, 13th, Left, Shortened

Litter 75 Foetus R02 RIB, 13th, Left, Shortened

Litter 75 Foetus R04 RIB, 13th, Left, Shortened

Litter 77 Foetus R01 THORACIC VERT.ARCH, 7th, Left, Aplasia, THORACIC VERT.CENTRA, 6th /7th, Fragmented,

THORACIC VERT.CENTRA, 6th and 7th, Left, Fused, RIB, 7th, Left, Aplasia

Litter 77 Foetus R05 THORACIC VERT.ARCH, 2nd, Right, Aplasia, THORACIC VERT.CENTRA, 2nd, Aplasia

STERNEBRA, 4th, Fragmented & Longitudinally displaced, RIB, 2nd, Right, Aplasia

Litter 79 Foetus R03 RIB, 4th, Bilateral, Thickened, RIB, 5th, Left, Thickened, RIB, 6th-7th, Bilateral, Thickened,

RIB, 8th-9th, Right, Thickened, RIB, 11th, Bilateral, Wavy

Litter 79 Foetus R07 THORACIC VERT.ARCH, 9th, Bilateral, Aplasia, THORACIC VERT.CENTRA, 9th, Aplasia

STERNEBRA, 4th, Longitudinally displaced, RIB, 5th-7th, Right, Thickened, RIB, 9th, Right, Aplasia,

RIB, 8th and 9th, Left, Fused, proximal part

Litter 79 Foetus L01 RIB, 3rd-8th, Bilateral, Thickened, RIB, 9th-11th, Bilateral, Wavy

Litter 79 Foetus L03 RIB, 4th-7th, Bilateral, Thickened

Litter 84 Foetus R02 THORACIC VERT.CENTRA, 11th, Fragmented

Litter 84 Foetus R04 THORACIC VERT.CENTRA, 13th, Fragmented

Litter 84 Foetus R06 THORACIC VERT.ARCH, 6th-7th, Right, Fused, THORACIC VERT.CENTRA, 6th, Dislocated

Litter 84 Foetus L05 THORACIC VERT.CENTRA, 11th, Dislocated

Litter 88 Foetus L03 THORACIC VERT.CENTRA, 12th, Fragmented

Litter 88 Foetus R06 STERNEBRA, 4th, Longitudinally displaced

Litter 89 Foetus R07 RIB, 9th-11th, Right, Thickened

The data show that 18 (12.7%) foetuses from 8 (38.1%) high dose litters showed minor defect/s of the thoracic region and an effect due to treatment is indicated. However, the nature of the minor defects is not indicative of permanent structural change detrimental to the animal i.e. malformation (according to OECD TG 414) and therefore, are considered not to have adverse consequences for the foetuses in postnatal life. The observations represent a perturbation of ossification, transient in nature, being resolved as ossification progresses.

Fluopicolide tested at 700 mg/kg bw/day, induced maternal toxicity in terms of reduced body weight gain particularly following the onset of dosing (↓ 24% of controls days 7-10). It is considered likely that the early development of the rat foetuses was impeded by the magnitude of the maternal effect between gestation days 7-10. However, as the minor skeletal anomalies were not indicative of permanent structural change or abnormality, it is considered that they should be considered non-adverse such that classification of fluopicolide as a developmental toxicant is not warranted. This conclusion is supported by reference to the CLP guidance[6] which states that 'classification is not necessarily the outcome in the case of minor developmental changes, when there is only a small reduction in foetal body weight or retardation of ossification when seen in association with maternal toxicity'.

It is also important to note that there is no reason to dispute the minor nature of the findings on the basis of the terminology used. Where the aplasia or dysplasia is considered to be a malformation, the term major defect is clearly ascribed (e.g. foetus L05 from litter 60). The performing laboratory has a clear structure for categorising foetal findings and the foetal data indicate that the criteria have been applied correctly.

Skeletal observations considered indicative of retarded development were also of increased incidence at the highest dose only. Their occurrence was consistent with the lower foetal body weight in this dose group. This is because the extent of foetal ossification depends to some extent on foetal size and it is a common observation that smaller foetuses (from larger litters) show an increased incidence of delayed ossification when compared with larger foetuses (from smaller litters) of the same dose group (OECD GD 43). Although statistically significant differences were attained at lower doses, the incidence of affected foetuses was not dose-related and no association with treatment was indicated.

The NOAEL for maternal toxicity and fetotoxicity was 60 mg/kg bw/day, based on slightly decreased body weight in dams and a reduction in mean foetal body weight and crown–rump length in foetuses at 700 mg/kg bw/day. Further evidence of fetotoxicity at this dose was increased incidences of minor defects of the thoracic vertebrae, sternebrae and ribs, as well as delayed ossification. Fluopicolide was not teratogenic in the prenatal developmental toxicity study in rats.

2. Rabbit

A study was conducted in order to determine the effects of fluopicolide on the maternal state of health, embryonic and foetal development when administered orally during pregnancy ([redacted] 2004; M-202513-02-1). The study design was based on international test guidelines (including OECD TG 414 adopted 1981). Although the study predates the current version of the guideline (adopted in 2018), there are no significant deviations; the treatment period is consistent with the current guideline requirement. However, the highest dose tested did induce more than 10% maternal deaths and maternal toxicity leading to abortion such that only 5 litters were available at term for examination which is inadequate for meaningful assessment.

Groups of 23 mated female Himalayan rabbits received fluopicolide by gavage in 1% methylcellulose at dose levels of 5, 20 or 60 mg/kg bw/day on Days 6-28 of pregnancy (Day 0: day of mating) and were terminated on Day 29 of pregnancy.

There was no clear indication from the preliminary study ([redacted] 2000; M-241192-01-1) that the highest dose of 60 mg/kg bw/day would not be tolerated. However, in the definitive study, three animals given 60 mg/kg bw/day were found dead and 15 animals of this group were killed after premature delivery from Day 22-29 of gestation. These animals showed decreased defecation, reduced hay consumption, hypoactivity, bristling coat, pultaceous faeces and discoloured urine. Body weight gains and food consumption were markedly decreased in the animals from the 60 mg/kg bw/day group.

With respect to the litters, dead foetuses were present in most premature deliveries. Mean foetal body weights, crown-rump lengths and placental weights were decreased in the 5 high dose group litters at term. Litter size, number of live and dead foetuses as well as sex ratios remained unaffected by the administration of the test compound. Likewise, incidences of early and late conceptuses undergoing resorption were unaffected. Morphological examination of the foetuses did not reveal any compound-related effects but the number of litters in the high dose group precluded meaningful evaluation.

The intermediate dose of 20 mg/kg bw/day is a no observed effect level (NOEL) for both maternal and developmental toxicity indicating the steepness of the dose-response. Whilst a dose level higher than 20 mg/kg bw/day has not been fully evaluated for developmental effects, the steepness of the dose-response for maternal effects makes selection of a dose that might be tolerated and induce some toxicity difficult to predict. Given that the generally accepted analytical tolerance for achieved concentration of test substance in dosing formulations is ± 15%, the lower tolerance value for 60 mg/kg bw/day is 51 mg/kg bw/day and the upper tolerance value for 20 mg/kg bw/day is 23 mg/kg bw/day. Should a dose level higher than 20 mg/kg bw/day be required for evaluation, it would need to be in the range of 24-50 mg/kg bw/day (mid-range value 37 mg/kg bw/day). Whether or not the mid-range value would induce maternal toxicity is unknown. Given the lack of evidence of fluopicolide to induce adverse developmental effects in the absence of maternal toxicity, the justification for further animal experimentation is questionable.

There are no grounds for classification for developmental toxicity on the basis of this study which should be considered a valid assessment of the potential of fluopicolide to induce developmental toxicity in the rabbit.

III. Conclusion

On the basis of the results of a two-generation reproduction study in the rat and prenatal developmental toxicity studies in rats and rabbits, fluopicolide should not be classified as a reproductive toxicant based on the ECHA Guidance on the application of the CLP criteria.

Assessment and conclusion by applicant:

This position paper was intended to assess the potential for classification of fluopicolide as a reproductive toxicant. It was concluded that fluopicolide should not be classified as a reproductive toxicant based on the ECHA Guidance on the application of the CLP criteria.

The following studies are currently in progress and will be submitted at the indicated timepoint.

Dossier node	Draft title	Study ID	Planned submission
KCA 5.6.2	Dose range finding developmental toxicity study in rabbits	TXAC0088	2nd Quarter 2021
KCA 5.6.2	Developmental toxicity study in rabbits	TXAC0089	2nd Quarter 2021

CA 5.7 Neurotoxicity studies

A preliminary acute and definitive acute and subchronic neurotoxicity studies in rats are available.

In the acute study, doses of 0 (control group 1), 10, 100 or 2,000 mg/kg bw by gavage to rats were chosen based on the preliminary dose-range finding study. No animals died, no signs were seen at routine observations and body weights, food consumption, food conversion efficiency and brain weights and dimensions were unaffected by treatment. Macropathological and histopathological examination of the tissues did not reveal any findings related to the administration of fluopicolide. At the neurobehavioral screening, the only treatment-related finding were low body temperatures recorded in animals receiving 2,000 mg/kg bw at Day 1 only. Therefore, signs of a direct neurotoxic potential were not evident in this study. The No Observed Effect Level (NOEL) on this study is considered to be 100 mg/kg bw.

In a subchronic study, 10 male and 10 female CD rats received fluopicolide orally, via the diet, at concentrations of 0, 200, 1,400 or 10,000 ppm for a treatment period of 13 weeks. No animals died during the study. Routine daily clinical signs, detailed weekly observations, FOB and motor activity assessments, brain weights (absolute) and measurements and neuropathology were unaffected by the test substance. Only adaptive changes occurred in the liver at 1,400 and 10,000 ppm and male-specific nephropathy occurred at 1,400 and 10,000 ppm. Thus, it is concluded that the administration fluopicolide to CD rats for 13 weeks via the diet at concentrations of up to 10,000 ppm (equivalent to 781 mg/kg bw/day in males and 866 mg/kg bw/day in females) did not result in any neurotoxicity. The No Observed Effect Level (NOEL) in this study was established at 200 ppm (equivalent to 15.0 mg/kg bw/day in males and 18.0 mg/kg bw/day in females).

Table 5.7- 1: Overview of neurotoxicity studies performed with fluopicolide

Method, guideline, deviation(s) (if any), species, strain, sex no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
Acute neurotoxicity study: Dose-range finding study None-guideline study GLP compliant Rat, CD strain 3/sex/group	Fluopicolide (purity 95.9%) Oral (gavage) Dose levels: 0, 10, 50, 500, 2,000 mg/kg bw (two groups used for high dose) Single dose Vehicle: 1% methylcellulose	2,000, 500 and 50 mg/kg bw Behavioural changes including hunched posture, occasional tremor, reduced arousal accompanied by decreased activity and rearing scores and decreased body temperature with a peak effect at 6 hours after dosing. Due to a missing concurrent control for the higher treatment groups, the small group size (3 sex/group) and the missing reproducibility of results in female animals (incidence and/or severity of effects in female animals in group 1 could not be reproduced in group 2) the significance of the results is limited.	[redacted] 2002; M-211178-01-1 KCA 5.7.1/01

Method, guideline, deviation(s) (if any), species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
Acute neurotoxicity study OECD 424 (1997) GLP compliant Rat, CD strain 10/sex/group	Fluopicolide (purity 95.9%) Oral (gavage) Dose levels: 0, 10, 100, 2,000 mg/kg bw Single dose Vehicle: 1% methylcellulose	There were no deaths. 2,000 mg/kg bw **No neurotoxicity effects.** ↓ body temperature on the day of dosing only (transient; both sexes) 100 mg/kg bw **No treatment-related effects.** 10 mg/kg bw **No treatment-related effects.**	[redacted] 2002, M-208046-01-1 KCA 5.7.1/02
Sub-chronic neurotoxicity study OECD 424 (1997) GLP compliant Rat, CD strain 10/sex/group	Fluopicolide (purity 97.8%) Oral (dietary administration) Dose levels: 0, 200, 1,400, 10,000 ppm Equivalent to: Males: 0, 15, 107, 781 mg/kg bw/day Females: 0, 18, 125, 866 mg/kg bw/day Daily for 13 weeks Vehicle: diet	There were no deaths. 10,000 ppm (781 and 866 mg/kg bw/day in males and females, respectively) **No neurotoxicity effects.** ↓ BW (up to 13%) and overall BWG (19% and 28% in males and females, respectively) Slightly ↓ overall food consumption (7% and 8% in males and females, respectively) Liver (adaptive change) – centrilobular hepatocytic hyper-trophy (10/10 males, 6/10 females) Kidney – tubular hyaline droplets (10/10 males; ↑ severity), interstitial inflammation (9/10 males; ↑ severity), granular casts (10/10 males; ↑ severity), cortical tubular dilatation (7/10 males), hyaline tubular casts (4/10 males) 1,400 ppm (107 and 125 mg/kg bw/day in males and females, respectively) **No neurotoxicity effects.** Slightly ↓ overall BWG (13% in females) Liver (adaptive change) – centrilobular hepatocytic hyper-trophy (9/10 males) Kidney – tubular hyaline droplets (10/10 males; ↑ severity), interstitial inflammation (5/10 males; ↑ severity), granular casts (2/10 males) 200 ppm (15 and 18 mg/kg bw/day in males and females, respectively) **No treatment-related adverse effects.**	[redacted] 2002, M-208051-01-1 KCA 5.7.1/03

BW: body weight
↓: decreased
BWG: body weight gain
↑: increased

CA 5.7.1 Neurotoxicity studies in rodents

Data Point:	KCA 5.7.1/01
Report Author:	[redacted]
Report Year:	2002
Report Title:	Dose range and time to peak effect in rats by acute oral administration AE C638206
Report No:	C021425
Document No:	M-211178-01-1
Guideline(s) followed in study:	US-EPA 712-C-98-238), OPPTS 870,6200 (1988)
Deviations from current test guideline:	As a dose-range finding study the study was not intended to comply with OECD guidelines.
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

The objective of this study was to verify suitable dosages and the period of peak effect on behavioural function of fluopicolide when given to CD rats by acute oral administration. All animals received only one administration of the test substance and were dosed following overnight starvation.

One group of 3 male and 3 female CD rats received fluopicolide via oral gavage administration, at a dosage of 2,000 mg/kg bw. These animals comprised Group 1 and functional observational battery (FOB) observations were performed on these animals before commencement and at 0.5, 1, 2, and 4 hours after dosing. Signs were apparent at the 4-hour observation and, therefore additional observations were performed at 6 and 8 hours after dosing. FOB observations for all subsequent groups were recorded before commencement and at 4, 5 and 6 hours after dosing.

The progress of the study was determined on the basis of the results seen. A second group of 3 males and 3 females were introduced at the same dosage as Group 1 to determine the response more clearly. These animals comprised Group 2. Further groups of 3 males and 3 females were treated at lower dosages of 500 mg/kg bw (Group 3) and 50 mg/kg bw (Group 4). Signs continued to be observed in the Group 4 animals. A second batch of animals was ordered and one group of 3 male and 3 female CD rats received fluopicolide at a dosage of 10 mg/kg bw (Group 5). A further group of 2 male and 2 female CD rats constituted a control group (Group 6) and received the vehicle (1% methylcellulose) alone. These two groups of animals ran concurrently, all other groups ran sequentially.

For each animal body weights were recorded on the day of allocation, on the day of dosing, daily throughout the observation period and each time an FOB examination was performed.

No animals died, no signs were seen at routine observations and bodyweights were unaffected by treatment.

The initial dosage chosen, 2,000 mg/kg bw, had a marked effect on the animals at 4 and 6 hours after dosing. Neurological signs included hunched posture, tremors, reduced activity and rearing in the arena and reduced body temperature. At the 8 hour FOB four of the six animals showed complete recovery and the remaining two only had low activity and rearing counts and reduced arousal. To determine the response more clearly a second set of animals was introduced at the same dosage. On this occasion the signs seen in the males were similar to those seen previously but females were relatively unaffected.

A further group of animals were treated at a lower dosage, 500 mg/kg bw. The males were affected by treatment to the same degree as seen at 2000 mg/kg bw. Females receiving a dosage of 500 mg/kg bw were generally considered to be unaffected by treatment at all time-points. However, activity levels were also slightly lower at 6 hours after dosing. Given the response seen in the males, it was necessary to treat an additional group of animals at a lower dosage of 50 mg/kg bw.

At 50 mg/kg bw, possibly treatment-related signs included low activity/rearing scores and tremor in some animals. Therefore, at 50 mg/kg bw there might be still an effect of treatment and it was decided to treat an additional group of animals at a lower dosage – 10 mg/kg bw. It was also decided to include control animals on this occasion to aid in the interpretation of the data.

At 10 mg/kg bw most males showed very low activity and rearing scores on the day of dosing when compared with pre-treatment values but similarly low scores were also seen in one of the two control males at 5 and 6 hours after dosing. This suggests that the low levels of exploratory activity were at least partly due to previous experience in the arena and cannot be confidently ascribed to treatment.

It is concluded that the single administration of fluopicolide to CD rats at dosages of 2,000, 500 and 50 mg/kg induced behavioural changes in animals. No clear effect was seen at 10 mg/kg bw. With the exception of the first 2,000 mg/kg bw administration, males were more severely affected than females. The time to peak effect was determined as 6 hours after dosing. However, due to a missing concurrent control for the higher treatment groups, the small group size (3 sex/group) and the missing reproducibility of results in female animals (incidence and/or severity of effects in female animals in group 1 could not be reproduced in Group 2) the significance of the results is limited.

A dose range of 10 mg/kg (low dose) to 2,000 mg/kg (top dose) was considered suitable to test in the definitive study.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 (fluopicolide)
Purity:	98.9% (w/w)
Batch no.:	OP2050046

2. Vehicle and/or positive control

Vehicle:	1% methylcellulose

3. Test animals

Species:	Rat
Strain:	Male and female CD rats
Age:	48-61 days at dosing
Weight at start:	Groups 1 to 4: males weighed 272 to 290 g and females weighed 187 to 198 g. The allocation weight ranges for the second batch of animals (Groups 5 and 6) were 211 to 226 g for the males and 159 to 169 g for the females
Source:	[redacted]
Acclimation period:	Yes
Diet:	Rat and Mouse No. 1 Maintenance Diet from Special Diets Services Ltd., Witham, Essex, England except overnight before dosing and continuing until the last FOB of the day had been performed.

Water:	Water taken from the public supply (Essex and Suffolk Water Company, Chelmsford, Essex, England), was freely available, via polycarbonate bottles fitted with sipper tubes.
Housing:	Modified RB3 cages from North Kent Plastic Cages Limited, Erith, Kent, England, which were made of a plastic body with a stainless steel mesh lid and floor
Temperature:	19-23 °C
Humidity:	40-70%
Air changes:	At least 15/hour
Photoperiod:	12 hours

B. Study design

1. In-life dates: January 10 to February 27, 2001

2. Animal assignment and treatment

Animals received the test material formulations by single oral gavage administration at a volume dosage of 10 mL/kg bw. Animals in Groups 1, 2, 3 and 4 were dosed in sequence of cage-number within each group. Animals in Groups 5 and 6 were dosed in the order in which the FOB observations were performed. The volume administered to each animal was calculated from the body weight measured immediately before the administration. Formulations were stirred with a magnetic stirrer before and throughout the dosing procedure.

All animals were starved overnight before dosing and the majority remained without food until after the last FOB observation was performed. For the Group 1 animals, food was available during the 8 hour FOB examination and for Groups 5 and 6 food was returned before the 6 hour FOB observation.

Animals were assigned to the groups as follows.

Table 5.7.1- 1: Study design

Group	Treatment	Dose (mg/kg bw)	Number of males	Number of females
1	Fluopicolide	2,000	3	3
2	Fluopicolide	2,000	3	3
3	Fluopicolide	500	3	3
4	Fluopicolide	50	3	3
5	Fluopicolide	10	3	3
6	Control	0	2	2
7*	Fluopicolide		3	3

* Animals were not treated and were discarded at the end of the study

The required amount of fluopicolide was weighed out and transferred to a suitably sized mortar and ground using a pestle. A small amount of 1% methylcellulose was added and mixed with the fluopicolide using the pestle to form a smooth paste. Further small amounts of 1% methylcellulose were added to form a smooth, pourable suspension. The suspension was added to a measuring cylinder that had been pre-rinsed with 1% methylcellulose and also contained a small amount of the vehicle in the bottom. Small amounts of 1% methylcellulose were added to the mortar and pestle to remove all traces of fluopicolide with the rinsing being added to the cylinder. The required volume was then made up in the cylinder using 1% methylcellulose and the suspension was transferred to a beaker, rinsing out the cylinder with the suspension to remove any concentrate and finally scraping out the cylinder. The suspension was then mixed using a high-shear homogeniser to further break down any agglomerates of fluopicolide and to produce an homogenous suspension.

The suitability of the proposed mixing procedure and the homogeneity and stability of the test substance in the vehicle at concentrations of 1 and 200 mg/mL was assessed in association with the forthcoming single dose rat neurotoxicity study (AES/046). Fluopicolide produced a homogeneous suspension in the vehicle which could be maintained for up to 2 hours while magnetically stirred and which could also be successfully re-suspended following ambient temperature storage for 2 days and refrigerated storage (4 °C) for 15 days.

Samples of each dose prepared were taken and stored frozen (-20 °C) pending any possible future requirement for analysis. The samples taken from the Group 3 formulation were initially stored at 4 °C and then transferred to -20 °C storage two days later. This incorrect storage of the samples for a short period of time is not considered to have affected the integrity of the study and, if the samples are analysed in the future, this factor will be taken into account.

The return of food on these occasions was necessary because the Project Licence states that animals must not remain without food for longer than a 24 hour period. Due to the unexpected inclusion of a FOB observation at 8 hours after dosing on Day 1 for Group 1 and the late arrival of the dose in the animal unit for the Group 3 animals, the 24 hour time limit would have been exceeded if food had not been returned to the animals before the end of the FOB examination. Since on both occasions, food was returned to all the animals before the start of the last FOB examination it is not considered that these deviations from protocol procedure have affected the integrity of the study.

All animals received a single dose followed by at least seven days of observation.

C. Methods

1. Observations

Animals were inspected at least twice daily for evidence of reaction to treatment or ill health. Any deviations from normal were recorded at the time in respect of nature and severity, date and time of onset, duration and progress of the observed condition, as appropriate.

After each single dose, detailed observations were recorded before and after dosing; these observations were recorded at the following times in relation to dosing:

- Immediately before dosing.
- Immediately after dosing on return of the animal to its cage.
- 1-2 hours after dosing.
- Throughout the day observations were made as part of the functional observation battery screening examinations.
- As late as possible in the working day.

Cages and cage-trays were inspected daily for evidence of ill health, such as blood or loose faeces. During the acclimatization period, observations of the animals and their cages were made at least once per day.

2. Body weights

Each animal was weighed on the day of allocation, on the day of dosing and on Days 8 and 15 of the study. Body weights were also recorded before necropsy. Further weightings occurred as part of the neurobehavioural screening.

3. Neurobehavioural screening - functional observational battery

A functional observational battery (FOB) was performed on all animals before the day of treatment and at timed intervals after dosing. The first group of animals (Group 1) were assessed at 0.5, 1, 2, 4, 6 and 8 hours after dosing, to establish the time of peak effect, and subsequent groups were assessed at 4, 5 and 6 hours after dosing.

The functional observational battery and motor activity recordings were performed at approximately the same time of day on each occasion and when observing Groups 5 and 6 the observer was unaware of the experimental group to which the animal belonged. This was achieved by removing cage labels showing the treatment group and replacing them with labels stating only the study and animal numbers. The observer was aware of which group the animals belonged to for Groups 1 to 4 as only one group at a time was dosed. Any deviation from normal was recorded with respect to nature and, where appropriate, degree of severity.

Observations in the home cage:

The following parameters were assessed in the home cage, without disturbing the animals:

- Palpebral closure
- Posture
- Tremor
- Twitches
- Convulsions
- Spontaneous vocalization

Observations in the hand:

During and after removal from the home cage, the following parameters were assessed:

- Removal from cage
- Salivation
- Lacrimation
- Exophthalmos
- Piloerection
- Fur condition
- Vocalisation on handling
- Reactivity to handling

Observations in the arena:

The animal was placed in a standard arena (approx. 653 x 500 mm) with the floor marked into six sections of equal size for scoring activity counts (see below). The animal was observed for the following parameters during a 3-minute recording period:

- Arousal
- Gait
- Grooming
- Palpebral closure
- Posture
- Activity counts
- Rearing count
- Tremor, Twitches and Convulsions:
- Respiration
- Defaecation
- Urination

After completion of arena observations, the following measurements were made:

- Body temperature

4. Necropsy

Animals sacrificed at the end of the scheduled treatment period were killed by carbon dioxide inhalation and then discarded. Animals were not subjected to a formal necropsy and no tissues were retained or examined.

5. Statistics and evaluation

Group mean values were calculated from the individual values presented in the appendices, unless otherwise specified. The small sample size and absence of controls until the second consignment of animals precludes statistical evaluation.

II. Results and Discussion

A. Results

1. Clinical signs and mortality

No animals died as a result of the single administration of fluopicolide. There were no signs seen in the animals immediately after dosing and in the routine observations. Signs were apparent during the neurobehavioural screening which are discussed below.

2. Body weights and food consumption

The body weights of the animals were not affected by treatment. Large body weight gains were seen for all animals for the period Day 1-2. These are the result of the animals losing weight due to food deprivation before dosing and during the FOB investigations and subsequently compensating when the food was returned at the end of Day 1.

3. Neurobehavioural screening - functional observational battery

Home cage observations

4.0, 5.0 and 6.0 hours after dosing, hunched posture was seen in several animals with a higher incidence in the 500 mg/kg bw (males only) and 2000 mg/kg bw (both sexes) groups compared to the other treatment and the control group. In addition, palpebral closure was seen in a few animals of both sexes at 4 and 6 hours after dosing especially in the 2000 mg/kg bw groups. However, palpebral closure is not an unusual observation and assumed to be an indication of sleepiness at this time in the day which is supported by the fact that most of the animals had the additional comment of "animal appears asleep".

Table 5.7.1- 2: Home cage observations – incidence male animals

Parameter	Time interval [h]	Dose level [mg/kg bw]					
		2,000		500	50	10	0
		Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Animals/group		3	3	3	3	3	3
Palpebral closure	pre	0	0	1[A]	1[A]	0	0
	0.5	0	-	-	-	-	-
	1	1[A]	-	-	-	-	-
	2	2[A]	-	-	-	-	-
	4	1[A]	3	1	1[A]	0	0
	5	-	-	0	0	-	1
	6	2[A]	0	0	1	0	0
	8	0	-	-	-	-	-
Posture hunched	pre	0	0	0	0	-	-
	0.5	0	-	-	-	-	-
	1	0	-	-	-	-	-
	2	2	-	-	-	-	-
	4	1	3	3	2	0	1
	5	-	3	2	0	1	2
	6	3	3	1	0	0	0
	8	0	-	-	-	-	-
Tremor	pre	0	0	0	0	0	0
	0.5	0	-	-	-	-	-
	1	0	-	-	-	-	-
	2	0	-	-	-	-	-
	4	0	0	0	0	0	0
	5	0	0	0	0	0	0
	6	0	0	0	0	0	0
	8	0	-	-	-	-	-
Twitches	pre	0	0	0	0	0	0
	0.5	0	-	-	-	-	-
	1	0	-	-	-	-	-
	2	0	-	-	-	-	-
	4	0	0	0	0	0	0
	5	-	0	0	0	0	0
	6	0	0	0	0	0	0
	8	0	-	-	-	-	-
Convulsion	pre	0	0	0	0	0	0
	0.5	0	-	-	-	-	-
	1	0	-	-	-	-	-
	2	0	-	-	-	-	-
	4	0	0	0	0	0	0
	5	-	0	0	0	0	0
	6	0	0	0	0	0	0
	8	0	-	-	-	-	-
Vocalisation	pre	0	0	0	0	0	0
	0.5	0	-	-	-	-	-
	1	0	-	-	-	-	-
	2	0	-	-	-	-	-
	4	0	0	0	0	0	0
	5	-	0	0	0	0	0
	6	0	0	0	0	0	0
	8	0	-	-	-	-	-

A one animal appears asleep

Table 5.7.1- 3: Home cage observations – incidence female animals

Parameter	Time interval [h]	Dose level [mg/kg bw]					
		2,000		500	50	10	0
		Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Animals/group		3	3	3	3	3	3
Palpebral closure	pre	0	0	0	0		
	0.5	0	-	-	-		-
	1	1[A]	-	-	-		-
	2	0	-	-	-	-	
	4	2[B]	0	1[A]	0	0	0
	5	-	0	0	0	0	1
	6	2[B]	1[A]	1	0	0	0
	8	0	-	-		-	-
Posture hunched	pre	0	0	0	0	0	0
	0.5	0	-	-	-		-
	1	0	-	-	-	-	
	2	0	-	-		-	-
	4	1	1	1	0	0	0
	5	-	0	0	0	1	2
	6	3	0	0	0	0	0
	8	0	-	-	-	-	-
Tremor	pre	0	0	0	0	0	0
	0.5	0	-	-	-	-	-
	1	0	-	-	-	-	-
	2	0	-	-	-	-	-
	4	0	0	0	0	0	0
	5	0	0	0	0	0	0
	6	0	0	0	0	0	0
	8	0	-	-	-	-	-
Twitches	pre	0	0	0	0	0	0
	0.5	0	-	-	-	-	-
	1	0	-	-	-	-	-
	2	0	-	-	-	-	-
	4	0	0	0	0	0	0
	5	-	0	0	0	0	0
	6	0	0	0	0	0	0
	8	0	-	-	-	-	-
Convulsion	pre	0	0	0	0	0	0
	0.5	0	-	-	-	-	-
	1	0	-	-	-	-	-
	2	0	-	-	-	-	-
	4	0	0	0	0	0	0
	5	-	0	0	0	0	0
	6	0	0	0	0	0	0
	8	0	-	-	-	-	-

Parameter	Time interval [h]	Dose level [mg/kg bw]					
		2,000		500	50	10	0
		Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Vocalisation	pre	0	0	0	0	0	0
	0.5	0	-	-	-	-	-
	1	0	-	-	-	-	-
	2	0	-	-	-	-	-
	4	0	0	0	0	0	0
	5	-	0	0	0	0	0
	6	0	0	0	0	0	0
	8	0	-	-	-	-	-

A One animal appears asleep
B Two animals appear asleep

In the hand observations

At 0.5, 1, 2 and 6 hours after dosing a whole body tremor was recorded in one female at 2,000 mg/kg bw and at 6 hours after dosing in one male at 500 mg/kg bw. However, in males there was no clear dose-dependency and in females the tremors were only observed in the first 2,000 mg/kg bw group. No other treatment-related effects or changes were observed at any dose level.

Table 5.7.1- 4: In the hand observations – incidence male animals

Parameter	Time interval [h]	Dose level [mg/kg bw]					
		2,000		500	50	10	0
		Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Animals/group		3	3	3	3	3	2
Salivation	pre	1	0	0	0	0	0
	0.5	1	-	-	-	-	-
	1	1	-	-	-	-	-
	2	0	-	-	-	-	-
	4	1	0	0	0	0	0
	5	-	0	1	0	0	0
	6	0	0	0	0	0	0
	8	0	-	-	-	-	-
Lacrimation	pre	0	0	0	0	0	0
	0.5	0	-	-	-	-	-
	1	0	-	-	-	-	-
	2	0	-	-	-	-	-
	4	0	0	0	0	0	0
	5	-	0	0	0	0	0
	6	0	0	0	0	0	0
	8	0	-	-	-	-	-
Piloerection	pre	0	0	0	0	0	0
	0.5	0	-	-	-	-	-
	1	0	-	-	-	-	-
	2	0	-	-	-	-	-
	4	0	0	0	0	1	1
	5	-	0	0	0	2	1
	6	0	0	0	0	0	0
	8	0	-	-	-	-	-

Parameter	Time interval [h]	Dose level [mg/kg bw]					
		2,000		500	50	10	0
		Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Exophthalmos	pre	0	0	0	0	0	0
	0.5	0	-	-	-	-	-
	1	0	-	-	-	-	-
	2	0	-	-	-	-	-
	4	0	0	0	0	0	0
	5	-	0	0	0	0	0
	6	0	0	0	0	0	0
	8	0	-	-	-	-	-
Brown fur staining	pre	1	0	1	2	0	2
	0.5	1	-	-	-	-	-
	1	0	-	-	-	-	-
	2	0	-	-	-	-	-
	4	0	0	2	2	0	2
	5	-	0	1	3	0	2
	6	0	1	2	2	0	2
	8	0	-	-	-	-	-
Hair loss	pre	0	0	0	0	0	0
	0.5	1	-	-	-	-	-
	1	1	-	-	-	-	-
	2	1	-	-	-	-	-
	4	1	1	0	0	0	0
	5	-	1	0	0	0	0
	6	1	-	0	0	0	0
	8	1	-	-	-	-	-
Vocalisation	pre	0	0	0	0	0	0
	0.5	1	-	-	-	-	-
	1	2	-	-	-	-	-
	2	0	-	-	-	-	-
	4	0	0	0	0	0	0
	5	-	0	0	0	0	0
	6	0	0	0	0	0	1
	8	0	-	-	-	-	-
Occassional, moderate whole body tremor	pre	0	0	0	0	0	0
	0.5	0	-	-	-	-	-
	1	0	-	-	-	-	-
	2	0	-	-	-	-	-
	4	0	0	0	0	0	0
	5	-	0	0	0	0	0
	6	0	0	1	0	0	0
	8	0	-	-	-	-	-

Table 5.7.1- 5: In the hand observations – incidence female animals

Parameter	Time interval [h]	Dose level [mg/kg bw]					
		2,000		500	50	10	0
		Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Animals/group		3	3	3	3	3	3
Salivation	pre	0	0	0	0	0	0
	0.5	0	-	-	-	-	-
	1	0	-	-	-	-	-
	2	0	-	-	-	-	-
	4	0	0	0	0	0	0
	5	-	0	0	0	0	0
	6	0	0	0	0	0	0
	8	0	-	-	-	-	-
Lacrimation	pre	0	0	0	0	0	0
	0.5	0	-	-	-	-	-
	1	0	-	-	-	-	-
	2	0	-	-	-	-	-
	4	0	0	0	0	0	0
	5	-	0	0	0	0	0
	6	0	0	0	0	0	0
	8	0	-	-	-	-	-
Piloerection	pre	0	0	0	0	0	0
	0.5	0	-	-	-	-	-
	1	0	-	-	-	-	-
	2	0	-	-	-	-	-
	4	0	0	0	0	0	0
	5	0	0	0	0	0	0
	6	0	0	0	0	0	0
	8	0	-	-	-	-	-
Exophthalmos	pre	0	0	0	0	0	0
	0.5	0	-	-	-	-	-
	1	0	-	-	-	-	-
	2	0	-	-	-	-	-
	4	0	0	0	0	0	0
	5	-	0	0	0	0	0
	6	0	0	0	0	0	0
	8	0	-	-	-	-	-
Brown fur staining	pre	2	2	2	2	1	1
	0.5	3	-	-	-	-	-
	1	3	-	-	-	-	-
	2	3	-	-	-	-	-
	4	3	2	3	3	3	2
	5	-	1	3	3	3	2
	6	3	0	3	3	2	2
	8	3	-	-	-	-	-
Hair loss	pre	0	0	0	0	0	1
	0.5	0	-	-	-	-	-
	1	0	-	-	-	-	-
	2	0	-	-	-	-	-
	4	0	0	0	0	0	1
	5	-	0	0	0	0	1
	6	0	0	0	0	0	1
	8	0	-	-	-	-	-

Parameter	Time interval [h]	Dose level [mg/kg bw]					
		2,000		500	50	10	0
		Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Vocalisation	pre	0	0	3	2	2	1
	0.5	2	-	-	-	-	-
	1	0	-	-	-	-	-
	2	0	-	-	-	-	-
	4	0	0	2	2	1	1
	5	-	0	2	2	0	1
	6	0	0	0	2	1	1
	8	0	-	-	-	-	-
Occassional, moderate, whole body tremor	pre	0	0	0	0	0	0
	0.5	1	-	-	-	-	-
	1	1	-	-	-	-	-
	2	1	-	-	-	-	-
	4	0	0	0	0	0	0
	5	-	0	0	0	0	0
	6	1	0	0	0	0	0
	8	0	-	-	-	-	-

Arena observations

Treatment-related reduced arousal was seen in male animals of the 500 mg/kg bw and both 2,000 mg/kg bw groups at 2, 4, 5 and 6 hours after treatment. In female animals the effect was less significant, and a clear reduction was only observed in the first 2,000 mg/kg bw group after 6 hours.

An increased incidence of tremors was seen in several animals of the 500 mg/kg bw (males only) and 2,000 mg/kg bw groups (both sexes) on several occasions. However, in males there was no clear dose-dependency and in females the tremors were only observed in the first 2,000 mg/kg bw group. Tremors were also seen in one male at 50 mg/kg bw and in one male at 10 mg/kg bw at 6 and 5 hours after treatment, respectively. Since tremor is occasionally seen also in control animals and since during the other examinations no tremors were observed at 10 mg/kg bw, the tremor in one male animal during the arena observations is rather attributed to the nervousness of the animal than to the substance treatment.

Slow respiration was observed in one single male animal in the first 2,000 mg/kg bw group after 4 and 6 hours. Effects on respiration were not seen in females or in males of the second 2,000 mg/kg bw group or any other dose group. Therefore, a treatment-relation is not assumed.

Activity and rearing scores were markedly reduced in male animals of the 50, 500 and both 2,000 mg/kg bw groups at 2, 4, 5 and 6 hours after treatment. At 10 mg/kg bw most males also showed very low activity and rearing scores on the day of dosing when compared with pre-treatment values, but similarly low scores were also seen in one of the two control males at 5 and 6 hours after dosing. This suggests that the low levels of exploratory activity were at least partly due to previous experience in the arena and cannot be confidently ascribed to treatment. It is suggested that as the study progressed the animals placed on study became older and the duration of individual housing increased. These factors need to be considered in the interpretation of the data.

Female animals were less affected with significantly activity and rearing reductions in the first 2,000 mg/kg bw group at 4, 5 and 6 hours and only slight effects in the 50, 500 and the second 2,000 mg/kg bw group at 6 hours. 8 hours after dosing, both sexes showed almost complete recovery.

Table 5.7.1- 6: Arena observations –male animals

Parameter	Time interval [h]	Dose level [mg/kg bw]					
		2,000		500	50	10	0
		Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Animals/group		3	3	3	3	3	3
Palpebral closure (incidence)	pre	0	0	0	0	0	0
	0.5	0	-	-	-	-	-
	1	0	-	-	-	-	-
	2	0	-	-	-	-	-
	4	1	1	0	1	0	0
	5	-	0	0	0	0	0
	6	0	0	1	0	0	0
	8	0	-	-	-	-	-
Arousal (mean grade 1-5)	pre	3.0	3.0	3.0	3.0	3.0	3.0
	0.5	2.7	-	-	-	-	-
	1	2.7	-	-	-	-	-
	2	2.0	-	-	-	-	-
	4	2.0	2.3	2.3	2.7	2.3	3.0
	5	-	2.0	2.0	2.3	2.3	2.5
	6	2.3	2.3	2.0	2.3	2.3	2.5
	8	2.7	-	-	-	-	-
Tremor (incidence)	pre	0	0	0	0	0	0
	0.5	0	-	-	-	-	-
	1	0	-	-	-	-	-
	2	0	-	-	-	-	-
	4	0	1	2	0	0	0
	5	2	2	3	0	1	0
	6	1	1	3	0	0	0
	8	0	-	-	-	-	-
Twitches/Convulsion (incidence)	pre	0	0	0	0	0	0
	0.5	0	-	-	-	-	-
	1	0	-	-	-	-	-
	2	0	-	-	-	-	-
	4	0	0	0	0	0	0
	5	-	0	0	0	0	0
	6	0	0	0	0	0	0
	8	0	-	-	-	-	-
Slow respiration (incidence)	pre	0	0	0	0	0	0
	0.5	0	-	-	-	-	-
	1	0	-	-	-	-	-
	2	0	-	-	-	-	-
	4	1	0	0	0	0	0
	5	-	0	0	0	0	0
	6	1	0	0	0	0	0
	8	0	-	-	-	-	-
Activity count (mean)	pre	10.7	19.0	15.0	16.3	12.7	22.5
	0.5	18.3	-	-	-	-	-
	1	10.7	-	-	-	-	-
	2	2.7	-	-	-	-	-
	4	1.3	3.3	4.3	6.0	8.7	17.5
	5	-	2.0	4.0	2.3	2.0	7.0
	6	7.7	4.3	1.7	2.7	10.0	8.0
	8	17.0	-	-	-	-	-

Parameter	Time interval [h]	Dose level [mg/kg bw]					
		2,000		500	50	10	0
		Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Rearing count (mean)	pre	10.3	15.3	18.0	13.7	10.3	15.5
	0.5	13.7	-	-	-	-	-
	1	7.3	-	-	-	-	-
	2	1.7	-	-	-	-	-
	4	0.3	5.0	1.3	5.3	3.7	13.0
	5	-	2.0	2.3	1.3	1.0	4.5
	6	3.3	3.7	1.0	2.3	8.3	3.0
	8	10.3	-	-	-	-	-

Table 5.7.1- 7: Arena observations –female animals

Parameter	Time interval [h]	Dose level [mg/kg bw]					
		2,000		500	50	10	0
		Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Animals/group		3	3	3	3	3	2
Palpebral closure (incidence)	pre	0	0	0	0	0	0
	0.5	0	-	-	-	-	-
	1	0	-	-	-	-	-
	2	0	-	-	-	-	-
	4	2	0	0	0	0	0
	5	-	0	0	0	0	0
	6	0	0	0	0	0	0
	8	0	-	-	-	-	-
Arousal (mean grade 1-5)	pre	3.0	3.0	3.0	3.0	3.0	3.0
	0.5	2.7	-	-	-	-	-
	1	2.7	-	-	-	-	-
	2	2.7	-	-	-	-	-
	4	2.7	3.0	3.0	2.7	3.0	3.0
	5	-	3.0	3.0	2.7	3.0	3.0
	6	2.0	2.7	3.0	2.7	3.0	3.0
	8	2.7	-	-	-	-	-
Tremor (incidence)	pre	0	0	0	0	0	0
	0.5	0	-	-	-	-	-
	1	1	-	-	-	-	-
	2	1	-	-	-	-	-
	4	2	0	0	0	0	0
	5	-	0	0	0	0	0
	6	3	0	0	0	0	0
	8	0	-	-	-	-	-
Twitches/Convulsion (incidence)	pre	0	0	0	0	0	0
	0.5	0	-	-	-	-	-
	1	0	-	-	-	-	-
	2	0	-	-	-	-	-
	4	0	0	0	0	0	0
	5	-	0	0	0	0	0
	6	0	0	0	0	0	0
	8	0	-	-	-	-	-

Parameter	Time interval [h]	Dose level [mg/kg bw]					
		2,000		500	50	10	0
		Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Slow respiration (incidence)	pre	0	0	0	0	0	0
	0.5	0	-	-	-	-	-
	1	0	-	-	-	-	-
	2	0	-	-	-	-	-
	4	0	0	0	0	0	0
	5	-	0	0	0	0	0
	6	0	0	0	0	0	0
	8	0	-	-	-	-	-
Activity count	pre	17.0	28.7	22.0	18.0	24.7	24.5
	0.5	18.3	-	-	-	-	-
	1	16.0	-	-	-	-	-
	2	13.7	-	-	-	-	-
	4	4.7	29.3	27.7	15.0	21.7	28.5
	5	-	22.3	22.0	13.3	12.3	21.5
	6	2.3	9.3	13.0	9.3	30.3	21.5
	8	18.7	-	-	-	-	-
Rearing count	pre	13.7	20.3	14.7	10.7	17.0	18.5
	0.5	16.7	-	-	-	-	-
	1	14.0	-	-	-	-	-
	2	14.0	-	-	-	-	-
	4	3.3	20.0	15.3	9.3	14.7	19.5
	5	-	17.3	13.7	10.3	11.0	24.5
	6	1.0	10.3	7.3	8.0	21.0	29.0
	8	15.0	-	-	-	-	-

Manipulation observations

When compared with the pre-treatment recordings significant reductions in body temperature were seen in male and female animals at 4 and 6 hours after dosing in the first 2,000 mg/kg bw group and at 4 hours only in the second 2,000 mg/kg bw group. When compared with the pre-treatment recordings significant reductions in body temperature were also seen in one male at 500 mg/kg bw at 4 and 6 hours after dosing. No effect on body temperature was observed in the lower treatment groups or in females at 500 mg/kg bw.

At 50, 500 and 2,000 mg/kg bw groups tremors were recorded in single male animals at 4, 5 and/or 6 hours after dosing during the handling procedure. There were no findings recorded during the handling procedure which were considered to be related to treatment at 10 mg/kg bw.

Table 5.7.1- 8: Mean body temperature

Parameter	Time interval [h]	Dose level [mg/kg bw]					
		2,000		500	50	10	0
		Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Males							
Body temperature (°C)	pre	37.8	37.5	37.6	37.3	37.6	37.7
	0.5	37.9	-	-	-	-	-
	1	37.8	-	-	-	-	-
	2	37.6	-	-	-	-	-
	4	37.0	36.4	36.7	37.2	37.2	37.5
	5	-	37.5	37.2	37.2	37.5	37.4
	6	37.3	37.1	36.9	37.3	38.3	38.1
	8	38.1	-	-	-	-	-
Females							
Body temperature (°C)	pre	37.7	37.8	37.5	37.3	37.5	37.5
	0.5	37.9	-	-	-	-	-
	1	37.9	-	-	-	-	-
	2	37.6	-	-	-	-	-
	4	36.9	37.2	37.3	37.6	37.8	37.7
	5	37.9	37.9	38.3	37.9	38.0[a]	38.2
	6	36.6	37.7	37.3	38.0	38.5[a]	38.6
	8	38.1	-	-	-	-	-

[a] calculated from only two animals

III. Conclusion

The single administration of fluopicolide to CD rats at dosages of 2,000, 500 and 50 mg/kg bw induced behavioural changes including hunched posture, occasional tremor, reduced arousal accompanied by decreased activity and rearing scores and decreased body temperature with a peak effect at 6 hours after dosing. No clear effect was seen at 10 mg/kg bw. With the exception of the first 2,000 mg/kg bw administration, males were more severely affected than females. However, due to a missing concurrent control for the higher treatment groups, the small group size (3 sex/group) and the missing reproducibility of results in female animals (incidence and/or severity of effects in female animals in group 1 could not be reproduced in group 2), the significance of the results is limited.

A dose range of 10 mg/kg (low dose) to 2,000 mg/kg (top dose) was considered suitable to test in the definitive study.

Assessment and conclusion by applicant:

The study was is valid to verify suitable dosages and the period of peak effect on behavioural function of fluopicolide when given to CD rats by acute oral administration. A dose range of 10 to 2000 mg/kg bw/d was selected for the definitive study.

Data Point:	KCA 5.7.1/02
Report Author:	[redacted]
Report Year:	2002
Report Title:	Neurotoxicity study by a single gavage administration to CD rats followed by a 14-day observation period AE C638206
Report No:	C019695
Document No:	M-208046-01-1
Guideline(s) followed in study:	US-EPA 712-C-98-238, OPPTS 870.6200 (1988)
Deviations from current test guideline:	Methods: none ;Study: Deviations from the current OECD guideline (424, 1997): None.
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

Groups of ten males and ten females received fluopicolide via oral gavage administration, at dosages of 0 (control group 1), 10, 100 or 2,000 mg/kg bw. The control group received the vehicle, 1% methylcellulose, at the same volume dosage as treated rats and served to generate contemporaneous control data.

Functional observational battery (FOB) observations were performed before commencement and on Day 1 (6 hours after dosing), 8 and 15. Body weights were recorded pre-treatment, immediately before dosing, on Days 8 and 15 and before necropsy. A body weight recording was also made each time an FOB was performed. Food consumption was recorded weekly. On completion of the study period the animals were sacrificed and examined macroscopically, the brain was measured for length and width and weighed. Selected tissues were retained for all animals and examined for five males and five females in the 0 and 2,000 mg/kg bw groups.

No animals died, no signs were seen at routine observations and body weights, food consumption, food conversion efficiency and brain weights and dimensions were unaffected by treatment.

Macropathological and histopathological examination of the tissues did not reveal any findings related to the administration of fluopicolide.

At the neurobehavioral screening, low body temperatures were recorded in animals receiving 2,000 mg/kg bw and a higher incidence of excessive grooming was seen in females receiving this dosage as compared with controls.

It is concluded that fluopicolide had a transient effect on body temperature in CD rats when given as a single oral gavage administration at a dosage of 2,000 mg/kg bw. The No Observed Effect Level (NOEL) on this study is considered to be 100 mg/kg bw.

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 (fluopicolide)
Purity:	95.9% (w/w)
Batch no.:	OP2050046

2. Vehicle and/or positive control

Vehicle:	1% methylcellulose

3. Test animals

Species:	Rat
Strain:	Male and female CD rats
Age:	42-48 days (males), 44-50 days (females) at dosing
Weight at start:	Males: 135 to 152 g, females: 118 to 138 g.
Source:	[redacted]
Acclimation period:	Yes
Diet:	Rat and Mouse No. 1 Maintenance Diet from Special Diets Services Ltd., Witham, Essex, England except overnight before dosing and continuing until the last FOB of the day had been performed.
Water:	Water taken from the public supply (Essex and Suffolk Water Company, Chelmsford, Essex, England), was freely available, via polycarbonate bottles fitted with sipper tubes.
Housing:	Modified RB3 cages from North Kent Plastic Cages Limited, Erith, Kent, England, which were made of a plastic body with a stainless steel mesh lid and floor
Temperature:	19-23 °C
Humidity:	40-70%
Air changes:	At least 15/hour
Photoperiod:	12 hours

B. Study design

1. In-life dates: March 21 to August 2, 2001

2. Animal assignment and treatment

During the pre-treatment period animals were weighed and ranked in order of body weight. Animals were then distributed amongst the treatment groups by body weight blocking to ensure that all groups contained populations of animals with similar initial mean and range of body weights. During the pre-treatment functional observational battery examinations it was noted that female No. 56 had severe ocular lesions. This animal was replaced with a spare female taken from the same batch of animals with a similar body weight.

As far as was practicable the distribution of animals in the room was designed to minimise the effect of any spatially variable component of the environment.

In addition, the cage batteries were rotated around the arrival room twice weekly. This ensured that each battery occupied different positions in the room during the course of the study.

Animals were assigned to the groups as follows.

Table 5.7.1- 9: Study design

Group	Treatment	Dose (mg/kg bw)	Number of males	Number of females
1	Vehicle control	0	10	10
2	Fluopicolide	10	10	10
3	Fluopicolide	100	10	10
4	Fluopicolide	2,000	10	10

The required amount of fluopicolide was weighed out and transferred to a suitably sized mortar and ground using a pestle. A small amount of 1% methylcellulose was added and mixed with the fluopicolide using the pestle to form a smooth paste. Further small amounts of 1% methylcellulose were added to form a smooth, pourable suspension. The suspension was added to a measuring cylinder that had been pre-rinsed with 1% methylcellulose and also contained a small amount of the vehicle in the bottom. Small amounts of 1% methylcellulose were added to the mortar and pestle to remove all traces of fluopicolide with the rinsing being added to the cylinder. The required volume was then made up in the cylinder using 1% methylcellulose and the suspension was transferred to a beaker, rinsing out the cylinder with the suspension to remove any concentrate and finally scraping out the cylinder. The suspension was then mixed using a high-shear homogeniser to further break down any agglomerates of fluopicolide and to produce an homogenous suspension.

Each dose was prepared and divided into four aliquots, one for each day of dosing. Doses were stored in a refrigerator (approx. 4 °C) and issued to the animal unit as required.

Before treatment the suitability of the proposed mixing procedure and the homogeneity and stability of the test substance in the vehicle at concentrations of 1 and 200 mg/mL was assessed.

The mean concentration of fluopicolide in the formulations prepared for dosing ranged from 89.8% to 94.0% of nominal concentrations and was considered satisfactory.

Animals received the test substance or control formulations by single oral gavage administration at a volume dosage of 10 mL/kg bw. Animals were dosed in four batches over four days, in the order in which the FOB observations were performed. The volume administered to each animal was calculated from the body weight measured immediately before the administration. Formulations were inverted approx. 20 times before dosing. They were also stirred with a magnetic stirrer before and throughout the dosing procedure.

All animals were starved overnight before dosing and remained without food until after the last FOB observation of the day was performed.

All animals received a single dose followed by fourteen days of observation.

C. Methods

1. Observations

Animals were inspected at least twice daily for evidence of reaction to treatment or ill health. Any deviations from normal were recorded at the time in respect of nature and severity, date and time of onset, duration, and progress of the observed condition, as appropriate.

After each single dose, detailed observations were recorded before and after dosing; these observations were recorded at the following times in relation to dosing:

- Immediately before dosing.
- Immediately after dosing on return of the animal to its cage.
- 1-2 hours after dosing.
- Throughout the day observations were made as part of the functional observation battery screening examinations.
- As late as possible in the working day.

Cages and cage-trays were inspected daily for evidence of ill health, such as blood or loose faeces. During the acclimatisation period, observations of the animals and their cages were made at least once per day.

2. Body weights and food consumption

Each animal was weighed on the day of allocation, on the day of dosing and on Days 8 and 15 of the study. Body weights were also recorded before necropsy. Further weightings occurred as part of the neurobehavioral screening.

The weight of food supplied to each cage, that remaining and an estimate of any spilled was recorded for the two weeks of the study. From these records the weekly consumption per animal was calculated. Food conversion efficiencies were calculated for each sex/group for the two weeks of treatment.

3. Neurobehavioral screening - functional observational battery

The functional observational battery (FOB) and motor activity examinations were performed on all animals before the commencement of treatment, on Day 1 at six hours after dosing and on Days 8 and 15 of the study. Any deviation from normal was recorded with respect to nature and, where appropriate, degree of severity.

The observer was unaware of the treatment of the individual animal because the animal numbering system was such that it was difficult to identify a treatment group by just the animal number. Prior to the observations the animal unit staff ensured that the labels on the cages gave the animal/cage number and contained no information on the group number, test material or dosages. This was recorded in the daybook as was the reversion to full cage labels after the observations were completed. In this study all animals were observed. In order to reduce bias during the observations, the animals were also examined in a random cage order.

<u>Observations in the home cage:</u>

The following parameters were assessed in the home cage, without disturbing the animal:

Convulsions, tremors, twitches
Palpebral closure
Posture
Spontaneous vocalisations

Observations in the hand:

During and after removal from the home cage, the following parameters were assessed:

Ease of removal from the cage
Exophthalmos
Fur condition
Piloerection
Reactivity to handling
Salivation/lacrimation
Vocalisation on handling

Observations in the arena:

The animal was placed in a standard arena (approx. 653 x 500 mm) with the floor marked into six sections of equal size for scoring activity counts (see below). The animal was observed for the following parameters during a 3-minute recording period.

Activity counts
Arousal
Tremor, Twitches and Convulsions
Defecation count
Gait
Grooming
Palpebral closure
Posture
Rearing count
Respiration
Urination

Manipulations:

After completion of arena observations, the following measurements, reflexes and responses were recorded:

Approach response
Body temperature
Body weight
Grip strength - fore and hindlimbs
Landing footspray
Pupil reflex
Righting reflex
Startle response
Tail pinch response
Touch response

Motor activity:

Motor activity was measured by automated infra-red equipment, recording individual animal activity at regular intervals over a one-hour period. The infra-red beams were arranged at two levels to record rearing and cage floor activity.

During the testing of the females, one of the stations on the activity monitoring equipment had a beam malfunction and could not be used. This resulted in two females, Nos. 43 and 79, not being tested. These females were from different treatment groups and the failure to record motor activity data for these animals does not prevent meaningful inter-group comparisons for this parameter. The overall validity of the study was not, therefore, compromised.

4. Necropsy

Animals sacrificed at the end of the scheduled treatment period were killed by an overdose of barbiturate by intra-peritoneal injection and exposure of the heart to permit perfusion with glutaraldehyde (1.5%):paraformaldehyde (4%) fixative via the aorta.

Macroscopic pathology:

All animals were subjected to a detailed necropsy. The necropsy procedures included a review of the history of each animal and a detailed examination of the external features and orifices, the neck and associated tissues and the cranial, thoracic, abdominal, and pelvic cavities and their viscera. The requisite organs were measured and weighed and external and cut surfaces of the organs and tissues were examined as appropriate. Abnormalities and interactions were noted, and the required tissue samples preserved in fixative.

Anatomical measurements:

The brain from each animal was transected from the spinal cord above the first cervical spinal nerve and the olfactory lobes removed. Measurements were taken of the length between the rostral part of the cerebral hemispheres to the most caudal part of the cerebellum and also the width between the widest parts of the cerebral hemispheres.

Brain weights:

The brain, taken from each animal, was dissected free of adjacent fat and other contiguous tissue and the weight recorded.

5. Pathology

Tissues preserved for histopathology:

Samples of the following tissues were preserved in glutaraldehyde (1.5%):paraformaldehyde (4%) fixative by *in situ* perfusion followed by immersion:

Brain	Skeletal muscle - gastrocnemius, right
Dorsal root fibres - cervical and lumbar	Spinal cord
Dorsal root ganglia - cervical and lumbar	Tibial nerves - at knee (right)
Eyes	Tibial nerve - calf muscle branch (es), (right)
Optic nerves	Ventral root fibres - cervical and lumbar
Sciatic nerve (right)	

Tissues preserved but not examined:

Samples of the tissues listed below were not processed histologically, but are held in fixative against any future requirement for microscopic examination:

Sciatic nerve, left *(in situ*)*
Skeletal muscle - gastrocnemius, left
Tibial nerve - at knee, left *(in situ*)*
Tibial nerve - calf muscle branch(es), left *(in situ*)*

* The carcass was retained.

Histology:

Tissue samples were taken from the five male and five female rats with the lowest animal numbers from the control and high dosage groups (Groups 1 and 4) sacrificed on completion of the treatment period and were processed as outlined below:

Using paraffin wax embedding procedures, sectioned at four to five micron thickness and stained with haematoxylin and eosin:

Brain: Six sections including all major regions (three cross sections of the forebrain, one cross section of each of the mid-brain, cerebellum and pons and medulla oblongata).
Spinal cord: Transverse and longitudinal sections at cervical and lumbar swellings.
Dorsal root ganglia: One cervical and one lumbar.
Dorsal root fibres: One longitudinal section at one cervical level, and at one lumbar level.
Ventral root fibres: One longitudinal section at one cervical level, and at one lumbar level.
Eyes (retina): One longitudinal section of each.
Optic nerves: One longitudinal section of each.
Skeletal muscle (gastrocnemius): One transverse section.
For eyes and optic nerves, sections of both the left and right organs were examined.

In addition, tissue samples from all animals sacrificed on completion of the scheduled treatment period were processed: Using resin embedding procedures, sectioned at approx. 2 micron thickness and stained with toluidine blue:

Sciatic nerve: Longitudinal and transverse sections at the sciatic notch and the mid-thigh.
Tibial nerve: Longitudinal and transverse sections at the knee and of calf muscle branch(es).

Microscopy:

Microscopic examination was performed as follows:

The tissues specified above were examined for the five male and five female rats with the lowest animal numbers from Groups 1 and 4 sacrificed on completion of the scheduled treatment period.

Findings were either reported as "Present" or assigned a severity grade. In the latter case one of the following five grades was used - minimal, slight, moderate, marked or severe.

The initial examination was undertaken by the Study Pathologist. The results of this examination were then subjected to a routine peer review by a second pathologist. The diagnosis reported here represent consensus opinions of both pathologists.

6. Statistics and evaluation

For body weight change, if 75% of the data (across all groups) were the same value, c, say, then a frequency analysis was applied. Treatment groups were compared using a Mantel test for a trend in proportions and also pairwise Fisher's Exact tests for each dose group against the control both for i) values <c versus values >=c, and for ii) values <=c versus values >c, as applicable.

If Bartlett's test for variance homogeneity was not significant at the 1% level, then parametric analysis was applied. If the F1 test for monotonicity of dose-response was not significant at the 1% level, Williams test for a monotonic trend was applied. If the F1 test was significant, suggesting that the dose-response was not monotone, Dunnett's test was performed instead.

If Bartlett's test was significant at the 1% level, then logarithmic and square root transformations were tried. If Bartlett's test was still significant, then non-parametric tests were applied. If the H1 test for monotonicity of dose-response was not significant at the 1% level, Shirley's test for a monotonic trend was applied. If the H1 test was significant, suggesting that the dose-response was not monotone, Steel's test was performed instead.

For brain weights changes, homogeneity of variance was tested using Bartlett's test. Whenever this was found to be statistically significant a Behrens-Fisher test was used to perform pairwise comparisons, otherwise a Dunnett's test was used.

The FOB numerical data were subjected to statistical analysis: activity and rearing in the standard arena, body temperature, body weight, landing foot splay, grip strength and motor activity. The following statistical analyses were performed:

> If the data consisted predominantly of one particular value (relative frequency of the mode exceeds 75%), the proportion of animals with different values from the mode was analysed using Fisher's Exact test. Otherwise, Bartlett's test was performed to test for variance heterogeneity between groups. Where significant (1% level) heterogeneity was found, the data were transformed, first logarithmically and if appropriate by square root, and re-tested for heterogeneity. If no statistically significant heterogeneity of variance was detected (with or without transformation), a one way analysis of variance was carried out. If the analysis of variance showed evidence (at the 5% level) of differences between the groups, Student's t test was used to test for differences between treatment groups and the control group. If heterogeneity was significant and could not be stabilised by transformation, the Kruskal-Wallis test on ranks was performed on the untransformed data. If the Kruskal-Wallis test showed evidence (5% level) of differences between the groups, the Wilcoxon Rank-Sum test was used to test for differences between the treatment groups and the control group.

Inter-group differences in macroscopic pathology and histopathology were assessed using Fisher's Exact test.

Unless stated, group mean values or incidences for the treated groups were not significantly different from those of the controls ($p > 0.05$).

II. Results and Discussion

A. Results

1. Clinical signs and mortality

No animals died as a result of the single administration of fluopicolide. There were no treatment-related signs seen in the animals immediately after dosing and in the routine observations.

2. Body weights and food consumption

The body weight gains of the animals were very variable both within and among the groups. There was no dosage-relationship in this variability and the bodyweight gains of the animals are considered to be unaffected by treatment.

Food consumption in the treated animals was similar to the controls. Food conversion efficiency was unaffected by treatment.

3. Neurobehavioral screening - functional observational battery

Observations in the home cage:

Home cage observations were unaffected by treatment.

Observations in the hand:

There were no differences considered to be associated with treatment.

Observations in the arena:

There were no observations noted in the rats during the time spent in the arena that were considered to be related to the administration of fluopicolide.

Increased grooming in females at 2,000 mg/kg on Day 1 was isolated in occurrence and thus considered to be of doubtful significance. Furthermore, the frequency of grooming was similar in all groups at Days 8 and 15 of the study, demonstrating that no permanent change in behaviour had been effected.

Females receiving 2,000 mg/kg showed a high incidence of elevated gait on the day of dosing, compared with controls, but the variable incidence in all treatment groups, including controls, on subsequent study occasions indicates that this was unlikely to represent an effect of treatment.

Males receiving 2,000 mg/kg showed an increased incidence of head shaking on the day of dosing, compared with controls (four versus none), but this behaviour was also observed in three control females and was not considered to be treatment-related.

FOB parameters were unaffected at Days 8 and 15.

Table 5.7.1- 10: Selected results following arena observations on Day 1

Parameter	Grade	Dose level (mg/kg bw)							
		Males				Females			
		0	10	100	2,000	0	10	100	2,000
Number of animals	-	10	10	10	10	10	10	10	10
Grooming	0	8	8	8	4	4	2	2	0
	1	2	2	2	6	6	8	7	6
	2	0	0	0	0	0	0	1	4
Elevated gait	-	3	0	2	3	0	2	2	7
Head shaking	-	0	0	1	4	3	1	0	1

Manipulations:

Compared with controls, a statistically significant reduction in body temperature was recorded in both males and females that had received 2,000 mg/kg bw, at the expected time of peak effect on the day of dosing. The temperature of most males in this group was between 36 and 37 °C but for some females it fell below 35 °C. Body temperature showed full recovery, with all groups showing similar group mean values on Days 8 and 15 of the study.

On Day 8 of the study, females that had received 2,000 mg/kg bw showed a statistically significant reduction in forelimb grip strength, compared with controls. Grip strength values for males and females were, however, unaffected on the day of dosing, and in the absence of reduced hindlimb values for females in these groups, or similar changes in males at Days 8 and 15, this effect was not considered to be associated with treatment.

Table 5.7.1- 11: Selected results following manipulation observations on Days 1, 8 and 15 (mean)

Parameter	Day	Dose level (mg/kg bw)							
		Males				Females			
		0	10	100	2,000	0	10	100	2,000
Number of animals	-	10	10	10	10	10	10	10	10
Body temperature (°C)	1	37.3	37.2	37.0	36.5***	36.9	36.8	36.8	36.0*
	8	37.5	37.4	37.4	37.4	37.4	37.3	37.3	37.5
	15	37.3	37.3	37.3	37.3	37.3	37.4	37.5	37.3
Fore limb grip strength (g)	1	561	557	586	555	559	534	523	523
	8	570	582	580	573	612	567	577	510**
	15	594	598	636	588	616	619	554	550
Hind limb grip strength (g)	1	203	189	208	194	204	196	185	190
	8	227	192	207	202	216	223	222	196
	15	225	198	212	209	212	220	220	204

* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$, statistically different to control

Motor activity:

Motor activity scores for males in all treated groups were statistically significantly lower than those of the controls on the day of dosing (Day 1), at the expected time of peak effect. Both high beam and low beam scores (rearing and cage floor activity, respectively) were similarly reduced. There was, however, not always a dosage-relationship and pre-treatment high and low beam scores for males in the treated groups were also lower than the controls (up to -25% and -18% for high and low beam scores, respectively) before commencement of the study. Taking both of these factors into account, the reduced activity scores shown by males in all treated groups on the day of dosing were not considered to be treatment-related.

Scores for females on the day of dosing (Day 1) showed considerable inter-group variation but there were no consistent difference considered to be associated with treatment. This further supports the absence of treatment-related findings in the male groups. Scores for both males and females on Days 8 and 15 of the study were considered unaffected.

Table 5.7.1- 12: Selected results following motor activity measurements on days 1, 8 and 15
(% difference to control)

	Beam	Dose level (mg/kg bw)					
		0	10		100		2,000	
Males								
Number of animals		10	10		10		10	
Pre-treatment	High	80.0	71.2	*(-11)*	65.9	*(-18)*	59.9	*(-25)*
	Low	471.4	420.8	*(-11)*	388.0	*(-18)*	416.8	*(-12)*
Day 1	High	111.9	71.6*	*(-36)*	78.4	*(-30)*	68.8*	*(-38)*
	Low	637.4	415.2*	*(-35)*	403.4**	*(-37)*	368.5**	*(-42)*
Day 8	High	133.7	118.2	*(-12)*	115.6	*(-13)*	109.7	*(-18)*
	Low	697.5	696.1	*(±0)*	693.3	*(-1)*	631.3	*(-9)*
Day 15	High	134.0	131.5	*(-2)*	118.0	*(-12)*	119.9	*(-10)*
	Low	714.9	680.4	*(-5)*	633.4	*(-11)*	604.8	*(-15)*

	Beam	Dose level (mg/kg bw)			
		0	10	100	2,000
Females					
Number of animals	-	10	10	10	10
Pre-treatment	High	65.3	68.6 *(+5)*	100.7 *(+54)*	77.2 *(+18)*
	Low	422.9	416.9 *(-1)*	500.3 *(+18)*	441.6 *(+4)*
Day 1	High	108.3	111.8 *(+3)*	91.6 *(-15)*	142.3 *(+31)*
	Low	626.1	626.6 *(±0)*	466.1 *(-25)*	520.9 *(-17)*
Day 8	High	117.7	132.7 *(+13)*	146.8 *(+25)*	151.4 *(+29)*
	Low	650.1	772.6 *(+19)*	757.6 *(+17)*	716.4 *(+10)*
Day 15	High	152.4	197.9 *(+30)*	156.9 *(+3)*	173.6 *(+14)*
	Low	762.2	996.2 *(+31)*	894.4 *(+17)*	872.5 *(+15)*

* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$, statistically different to control

4. Necropsy and pathology

Brain weights:

Brain weights were similar for control and treated animals.

Anatomical (cerebral hemisphere) measurements:

Measurements of the cerebral hemisphere did not reveal any differences in brain length or width between the control and treated animals.

Macro pathology:

Macropathological examination did not reveal any findings related to the administration of fluopicolide.

Histopathology:

There were no findings which were considered to be related to treatment.

III. Conclusion

The single oral gavage administration of fluopicolide to CD rats at dosages of 10, 100 or 2,000 mg/kg bw had no effects on body weights, food consumption, food conversion efficiency, brain weights and brain measurements. Macropathological and histopathological examination of the tissues did not reveal any findings related to the administration of the test substance.

The only notable observation related to treatment was seen in males and females at 2,000 mg/kg bw at the expected time of peak effect and consisted of low body temperatures.

It is concluded that fluopicolide had a transient effect on body temperature in CD rats when given as a single oral gavage administration at a dosage of 2,000 mg/kg bw. The No Observed Effect Level (NOEL) on this study is considered to be 100 mg/kg bw.

Assessment and conclusion by applicant:
An acceptable study yielding valid conclusions.

Data Point:	KCA 5.7.1/03
Report Author:	[redacted]
Report Year:	2002
Report Title:	Neurotoxicity study by dietary administration to CD rats for 13 weeks AE C638206
Report No:	C019700
Document No:	M-208051-01-1
Guideline(s) followed in study:	US-EPA 712-C-98-238). OPPTS 870.6200 (1988)
Deviations from current test guideline:	Deviations from the current OECD guideline (424, 1997): None.
Previous evaluation:	yes, evaluated and accepted DAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary:

Three groups of 10 male and 10 female CD rats received fluopicolide orally via the diet, at concentrations of 0, 200, 1,400 or 10,000 ppm for a treatment period of 13 weeks.

There were no deaths. The appearance and behaviour of the animals was unaffected by treatment. Overall body weight gains were low for males and females receiving 10,000 ppm and for females receiving 1,400 ppm and food consumption was slightly low for males and females receiving 10,000 ppm. The amount of food scattered was generally high for males and females receiving 1,400 or 10,000 ppm, and for females receiving 200 ppm, compared with the controls. Food conversion efficiency was low for males and females receiving 10,000 ppm and slightly low for females receiving 1,400 ppm. The overall achieved dosages at dietary concentrations of 200, 1,400 and 10,000 ppm were 15, 107 and 781 mg/kg bw/day for males and 18, 125 and 866 mg/kg bw/day for females.

The functional observational battery did not reveal any findings of significance. Absolute brain weights and anatomical measurements made of the cerebral hemispheres were not affected by treatment. Macroscopic examination revealed no treatment-related findings. There were no histopathological changes in the tissues presented for neuropathological examination which were considered to be related to treatment with fluopicolide.

However, treatment-related were seen in the liver and kidneys. An increased incidence of centrilobular hepatocyte hypertrophy of the liver which is considered adaptive was observed in males and females given 10,000 ppm and in males given 1,400 ppm. In addition, there was an increased incidence and/or severity of hyaline droplets in the cortical tubules in the kidneys of males at dose levels ≥1,400 ppm which was associated with increased incidences and severities of other degenerative or regenerative changes in the kidneys including inflammation, casts and dilatation. Hyaline droplets have been recognized as indicator of changes associated with the accumulation of α2μ-globulin in the cortical tubules of affected kidneys. The hyaline droplets in the cortical tubules of male rats are considered likely to represent accumulation of α2μ-globulin within the lysosomes. It is generally regarded that α2μ-globulin nephropathy is a male rat specific toxic response to the administration of certain types of chemicals (hydrocarbon nephropathy) and therefore not relevant for humans.

It is concluded that the administration of fluopicolide to CD rats for 13 weeks via the diet at concentrations of up to 10,000 ppm (equivalent to 781 mg/kg bw/day in males and 866 mg/kg bw/day in females) did not result in any neurotoxicity. Thus, the NOAEL for neurotoxicity was >10,000 ppm (equivalent to 781 mg/kg bw/day in males and 866 mg/kg bw/day in females). Adaptive changes occurred in the liver at 1,400 and 10,000 ppm and male rat-specific 'hydrocarbon nephropathy' occurred at 1,400 and 10,000 ppm. Therefore, the NOAEL for systemic toxicity in the 13-week neurotoxicity study was 200 ppm (15.0 mg/kg bw/day in males and 18.0 mg/kg bw/day in females) based on impaired growth and histopathological changes in kidney of male animals at dose levels of ≥1,400 ppm (equivalent 106.6 mg/kg bw/day in males and 125.2 mg/kg bw/day in females).

I. Materials and Methods

A. Materials

1. Test material

Test substance:	AE C638206 (fluopicolide)
Purity:	97.8%
Batch no.:	OP2050046

2. Vehicle and/or positive control

Vehicle:	Diet

3. Test animals

Species:	Rats
Strain:	CD strain
Age:	42 days old at start of study
Weight at start:	178 to 241 g (males, 151 to 183 g (females)
Source:	[redacted]
Acclimation period:	Yes
Diet:	Powdered rodent diet, Rat and Mouse No. 1 Maintenance Diet from Special Diets Services Ltd., Witham, Essex, England
Water:	Water taken from the public supply (Essex and Suffolk Water Company, Chelmsford, Essex, England), via polycarbonate bottles fitted with sipper tubes
Housing:	Individually housed in modified RB3 cages from North Kent Plastic Limited, Erith, Kent, England, which were made of a plastic body with a stainless steel mesh lid and floor.
Temperature:	19 – 23 °C
Humidity:	40 to 70%
Air changes:	At least 15/hour
Photoperiod:	12 hours

B. Study design

1. In-life dates: May 16 to December 12, 2001

2. Animal assignment and treatment

The rats were randomized and assigned to the following test groups.

Table 5.7.1- 13: Study design

Group no.	Dose (ppm)	Number of males	Number of females
1	0	10	10
2	200	10	10
3	1,400	10	10
4	10,000	10	10

The test substance was administered continuously via the diet throughout the treatment period. Animals did not have access to the formulated diet beyond its shelf life as determined by the stability assay.

Dietary concentrations of fluopicolide remained constant throughout the study.

Control animals received untreated diet at the same frequency and from the same batch of basal diet, as treated animals. The duration of treatment was 13 weeks.

Treatment, and the recording of serial observations, continued for all surviving animals throughout the necropsy period.

3. Diet preparation and analysis

Fluopicolide was incorporated into the ground diet to provide the required dietary concentrations by dilution of an appropriate premix. An initial premix at a concentration of 32,500 ppm was prepared.

The required amount of test substance was mixed with fine sieved diet by gentle stirring. Further quantities of sieved diet were then added to the premix until approximately half the required final weight was achieved. At this stage the premix was ground in a coffee grinder to ensure homogeneity of the mix. Coarse diet was added to make the premix up to the final required amount and mixed in a Turbula Mixer for 100 revolutions to ensure the test material was dispersed in the diet. Aliquots of this premix were then diluted with plain RM1 diet to produce the required highest and intermediate concentrations (1,400 and 10,000 ppm) for feeding to the animals and a second premix (3,250 ppm). The second premix was used to prepare the lowest concentration test mix (200 ppm). Each batch of treated diet was mixed for a further 100 revolutions in the Turbula mixer.

Batches of the test diets were prepared weekly and issued in sealed metal containers. The unused residues were discarded at the end of each treatment week. All diets were prepared at Huntingdon Research Centre and transported to Eye Research Centre for feeding to the animals.

The analytical procedure validation, the formulation homogeneity and the formulation stability, during both ambient temperature and frozen storage for 22 days, were confirmed for fluopicolide in rodent diet formulations at nominal concentrations of 50 and 2,500 ppm during an associated study.

Before commencement of treatment the homogeneity and stability of the test substance at 10,000 ppm (the highest concentration) in the diet was determined by a trial preparation. The homogeneity was assessed by analysing samples taken from the top, middle and bottom of the mix. The stability was determined after storage for 8 and 22 days at ambient temperature (nominally 21 °C). The results indicate that the test substance was homogeneous and stable for 22 days at ambient temperature.

Samples of each diet prepared for administration in Weeks 1 and 12 of treatment were analysed for the test substance. The results of these analyses demonstrated satisfactory achieved concentrations; the mean concentrations of fluopicolide in test diet formulations were within ±4% of nominal concentrations.

4. Statistics

For organ weights and body weight changes, homogeneity of variance was tested using Bartlett's test.

Whenever this was found to be statistically significant a Behrens-Fisher test was used to perform pairwise comparisons, otherwise a Dunnett's test was used.

Inter-group differences in macroscopic pathology and histopathology were assessed using Fisher's Exact test.

The functional observation battery numerical data were subjected to statistical analysis: activity and rearing in the standard arena, body temperature, bodyweight, landing foot splay, grip strength and motor activity.

The following statistical analyses were performed:

If the data consisted predominantly of one particular value (relative frequency of the mode exceeds 75%), the proportion of animals with different values from the mode was analysed using Fisher's Exact test. Otherwise, Bartlett's test was performed to test for variance heterogeneity between groups.

Where significant (1% level) heterogeneity was found, the data were logarithmically transformed and re-tested for heterogeneity. If no statistically significant heterogeneity of variance was detected (with or without logarithmic transformation), a one way analysis of variance was carried out. If the analysis of variance showed evidence (at the 5% level) of differences between the groups, Student's t-test was used to test for differences between treatment groups and the control group. If heterogeneity was significant and could not be stabilised by logarithmic transformation, the Kruskal-Wallis test on ranks was performed on the untransformed data. If the Kruskal-Wallis test showed evidence (5% level) of differences between the groups, the Wilcoxon Rank-Sum test was used to test for differences between the treatment groups and the control group.

Unless stated, group mean values or incidences for the treated groups were not significantly different from those of the controls ($p \geq 0.05$).

C. Methods

1. Observations

Animals were inspected at least twice daily for evidence of reaction to treatment or ill-health. Any deviations from normal were recorded at the time in respect of nature and severity, date and time of onset, duration and progress of the observed condition, as appropriate.

In addition, a more detailed weekly examination, which included palpation, was performed on each animal. Further observations were made as part of the neurobehavioral screening examinations.

Cages and cage-trays were inspected daily for evidence of ill-health, such as blood or loose faeces.

During the acclimatisation period, observations of the animals and their cages were recorded at least once per day.

2. Body weight and food intake

Each animal was weighed during the acclimatisation period (Week -1), on the day that treatment commenced (Week 0), at weekly intervals throughout the treatment period and before necropsy. Further weighing occurred as part of the neurobehavioral screening.

More frequent weighing was instituted, when appropriate, for animals displaying ill-health, so that the progress of the observed condition could be monitored.

The weight of food supplied to each cage, that remaining and an estimate of any spilled was recorded for each week throughout the treatment period. From these records the weekly consumption per animal was calculated.

Food conversion efficiencies were calculated for each sex/group for each week of treatment.

The group mean achieved dosage for each sex, expressed as mg/kg bw/day, was calculated at the same frequency that bodyweight was recorded. This was calculated from the nominal dietary test substance concentration, food consumption and bodyweight data.

3. Neurobehavioral screening - functional observational battery

Before commencement of treatment and during Weeks 4, 8 and 13 of treatment all animals were subjected to the procedures detailed below. The functional observational battery and motor activity recordings were performed at approximately the same time of day on each occasion and the observer was unaware of the experimental group to which the animal belonged. The animals were not necessarily all tested on the same day, but the number of animals were balanced across the groups on each day of testing. Any deviation from normal was recorded with respect to nature and, where appropriate, degree of severity.

4. Home cage observations

The animals were observed in the home cage for the following parameters:

- Convulsions, tremors, twitches
- Palpebral closure
- Posture
- Spontaneous vocalisations

5. In the hand and standard arena observations

Observations were performed in the hand and then during a three minute period in a standard arena.

After removal from the home cage the following parameters were assessed:

In the hand:	**Standard arena:**
Ease of removal from cage	Activity count
Exophthalmos	Arousal
Fur condition	Convulsion, tremors, twitches
Piloerection	Defecation count
Reactivity to handling	Gait
Salivation/lacrimation	Grooming
Vocalisation on handling	Palpebral closure
	Posture
	Rearing count
	Respiration
	Urination

6. Manipulations

The following measurements, reflexes and responses were recorded:

- Approach response
- Body temperature
- Body weight
- Grip strength - fore and hindlimbs
- Landing foot splay
- Pupil reflex
- Righting reflex
- Startle reflex
- Tail pinch response
- Touch response

7. Motor activity

Motor activity was measured by automated infra-red sensor equipment, recording individual animal activity over a one hour period.

8. Euthanasia

Animals were sacrificed by an overdose of barbiturate by intra-peritoneal injection and exposure of the heart to permit perfusion with glutaraldehyde/paraformaldehyde fixative via the aorta.

The sequence in which the animals were sacrificed after completion of the treatment period was selected to allow satisfactory inter-group comparison.

9. Pathology

Macroscopic pathology:

All animals were subjected to a detailed necropsy. The necropsy procedure included a review of the history of each animal and a detailed examination of the external features and orifices, the neck and associated tissues and the cranial, thoracic, abdominal, and pelvic cavities and their viscera. The requisite organs were measured and weighed and external and cut surfaces of the organs and tissues were examined as appropriate. Abnormalities and interactions were noted, and the required tissues samples preserved in fixative.

Organ weights:

The brain, taken from each animal, was dissected free of adjacent fat and other contiguous tissue and the weight recorded.

Anatomical measurements:

The brain from each animal was dissected from the spinal cord above the first cervical spinal nerve and the olfactory lobes removed. Measurements were taken of the length between the rostral part of the cerebral hemispheres to the most caudal part of the cerebellum and also the width between the widest parts of the cerebral hemispheres.

Tissues preserved for histopathology:

Samples of the following tissues were preserved in glutaraldehyde (1.5%) : paraformaldehyde (4%) fixative by *in situ* perfusion followed by immersion:

Brain	Spinal cord
Dorsal root fibres - cervical and lumbar	Tibial nerve - at knee, right
Dorsal root ganglia - cervical and lumbar	Tibial nerve - calf muscle branch (es), right
Eyes	Ventral root fibres - cervical and lumbar
Optic nerves	Adrenals
Sciatic nerve, right	Liver
Kidneys	Bone sections - stifle joint, femur, tibia and sternum
Skeletal muscle - gastrocnemius, right	

Samples of any abnormal tissues were also retained for histopathological examination.

Tissues preserved, but not examined:

Samples of the tissues listed below were not processed histologically, but held in fixative against any future requirement for microscopic examination:

Sciatic nerve, left (*in situ*)
Skeletal muscle - gastrocnemius, left
Tibial nerve - at knee, left (*in situ*)
Tibial nerve - calf muscle branch (es), left (*in situ*)

Histology:

Tissue samples taken from the five male and five female rats with the lowest animal numbers from the control and high dosage groups (Groups 1 and 4) sacrificed on completion of the treatment period were processed as outlined below:

Using paraffin wax embedding procedures, sectioned at four to five micron thickness and stained with haematoxylin and eosin:

Brain:	Three cross sections of the forebrain. One cross section of each of the mid-brain, cerebellum and pons and medulla oblongata.
Spinal cord:	Transverse and longitudinal sections at cervical and lumbar swellings.
Dorsal root ganglia:	One cervical and one lumbar.
Dorsal root fibres:	One longitudinal section at one cervical level and at one lumbar level.
Ventral root fibres:	One longitudinal section at one cervical level and at one lumbar level.
Eyes (retina):	One longitudinal section of each.
Optic nerves:	One longitudinal section of each.
Skeletal muscle (gastrocnemius):	One transverse section.

Using resin embedding procedures, sectioned at approx. 2 micron thickness and stained with toluidine blue:

Sciatic nerve:	Longitudinal and transverse sections at the sciatic notch and the mid-thigh.
Tibial nerve:	Longitudinal and transverse sections at the knee and calf muscle branch(es)

The following tissue samples taken all animals sacrificed on completion of the treatment period were processed as outlined below:

Using paraffin wax embedding procedures, sectioned at four to five micron thickness and stained with haematoxylin and eosin:

Adrenals:	Cortex and medulla
Stifle joint, femur and tibia:	Longitudinal section through joint to include articular surface, epiphyseal plate and bone marrow
Sternum:	Including bone marrow
Kidneys:	Including cortex, medulla and papilla regions
Liver:	Sections from all main lobes

Microscopy:

Microscopic examination was performed as follows:

- The brain, spinal cord, dorsal root ganglia, dorsal and ventral root fibres, eyes, optic nerves, skeletal muscle, sciatic nerve and tibial nerve were examined for the five male and five female rats with the lowest animal numbers from Groups 1 and 4.
- The adrenals, bone sections, kidney and liver were examined for all animals.

Findings were either reported as "Present" or assigned a severity grade. In the latter case one of the following five grades was used - minimal, slight, moderate, marked or severe.

The initial examination was undertaken by the Study Pathologist. The results of this examination were then subjected to a routine peer review by a second pathologist. The diagnosis reported here represents consensus opinions of both pathologists.

II. Results and Discussion

A. Results

1. Clinical results

The appearance and behaviour of the animals was unaffected by treatment. No animals died prematurely.

2. Body weight and food intake

Body weight

Overall body weight gains, when compared with the control, were significantly low for males and females receiving 10,000 ppm (81 and 72% of control values respectively; $p < 0.01$). Overall gains were also slightly low for females receiving 1,400 ppm (87% of control value, statistical significant).

Body weight gains for males and females receiving 200 ppm and for males receiving 1,400 ppm were not considered to be affected by treatment.

Table 5.7.1- 14: Selected body weight measurements (group means)

	Dose level (ppm)							
	Males				Females			
	0	200	1,400	10,000	0	200	1,400	10,000
Body weight [g] *(% difference to control)*								
Week 0	213	211 *(-1)*	207 *(-2)*	212 *(±0)*	167	171 *(+2)*	166 *(-1)*	172 *(+3)*
Week 1	268	275 *(+3)*	259 *(-3)*	243 *(-9)*	194	200 *(+3)*	190 *(-2)*	189 *(-3)*
Week 4	397	398 *(±0)*	378 *(-5)*	355 *(-11)*	245	252 *(+3)*	235 *(-4)*	235 *(-4)*
Week 8	497	493 *(-1)*	469 *(-6)*	434 *(-13)*	288	290 *(+1)*	273 *(-5)*	263 *(-9)*
Week 13	569	562 *(-1)*	536 *(-6)*	499 *(-13)*	318	316 *(-1)*	297 *(-7)*	281 *(-12)*
Body weight gain [g] *(% difference to control)*								
Week 0-13	356	351 *(-1)*	328 *(-8)*	287** *(-19)*	151	145 *(-4)*	131* *(-13)*	109** *(-28)*

* $p \leq 0.05$; ** $p \leq 0.01$, statistically different to control

Food intake

Overall food consumption, when compared with the controls, was slightly low for males and females receiving 10,000 ppm (93 and 92% of control values respectively).

Food consumption for males and females receiving 200 or 1,400 ppm was not affected by treatment.

Table 5.7.1- 15: Selected food consumption measurements (g/animal) *(% difference to control)*

	Dose level (ppm)							
	Males				Females			
	0	200	1400	10000	0	200	1400	10000
Week 1	205	211 *(+3)*	205 *(±0)*	179 *(-13)*	152	158 *(+4)*	157 *(+3)*	139 *(-9)*
Week 4	218	209 *(-4)*	204 *(-6)*	199 *(-9)*	146	160 *(+10)*	146 *(±0)*	142 *(-3)*
Week 8	224	219 *(-2)*	207 *(-8)*	196 *(-12)*	161	164 *(+2)*	157 *(-2)*	141 *(-12)*
Week 13	213	214 *(±0)*	208 *(-2)*	201 *(-6)*	159	153 *(-4)*	142 *(-11)*	134 *(-16)*
Total (week 0-13)	2834	2840 *(±0)*	2739 *(-3)*	2643 *(-7)*	2060	2148 *(+4)*	2010 *(-2)*	1885 *(-8)*

* $p \leq 0.05$; ** $p \leq 0.01$, statistically different to control

The amount of food scattered was generally higher than the controls for males and females receiving 1,400 or 10,000 ppm and for females receiving 200 ppm; males receiving 200 ppm were unaffected.

Individually housed animals are known to scatter more food than group housed animals and within the groups there was wide variability between the cages.

Food conversion efficiency was low, when compared with controls, for males and females receiving 10,000 ppm and slightly low for females receiving 1,400 ppm. There was no effect on food conversion efficiency for males and females receiving 200 ppm and for males receiving 1,400 ppm.

The achieved test substance doses were as follows.

Table 5.7.1- 16: Test substance doses (mg/kg bw/day)

Group no.	Dose (ppm)	Males	Females
2	200	15.0	18.0
3	1,400	106.6	125.2
4	10,000	780.6	865.8

3. Neurobehavioral screening (functional/observational battery report)

Home cage observations:

Home cage observations were unaffected by treatment.

In the hand observations:

Some inter-group variation occurred during in the hand observations but there were no differences considered associated with treatment.

Arena observations:

Arena observations showed some inter-group variation but there were no treatment-related changes.

Manipulations:

Body temperature values for females receiving 1,400 ppm were significantly increased compared with controls but as group mean values for females receiving 10,000 ppm were identical with controls on both occasions, this was not considered to be associated with treatment.

Table 5.7.1- 17: Body temperature measurements in Weeks 4, 8 and 13 (mean ± SD)

Parameter	Week	Dose level (ppm)							
		Males				Females			
		0	200	1,400	10,000	0	200	1,400	10,000
Number of animals	-	10	10	10	10	10	10	10	10
Body temperature (°C)	4	37.4 ± 0.4	37.5 ± 0.3	37.3 ± 0.2	37.3 ± 0.3	37.4 ± 0.2	37.6 ± 0.3	37.9*** ± 0.4	37.4 ± 0.2
	8	37.3 ± 0.3	37.4 ± 0.3	37.2 ± 0.2	37.3 ± 0.3	37.7 ± 0.4	37.7 ± 0.4	38.0 ± 0.5	37.5 ± 0.3
	13	37.3 ± 0.4	37.2 ± 0.2	37.1 ± 0.2	37.3 ± 0.3	37.6 ± 0.2	37.9 ± 0.4	38.1** ± 0.4	37.6 ± 0.3

* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$, statistically different to control
SD: Standard deviation

Motor activity:

Group mean motor activity scores showed no consistent dosage or treatment-related changes and none of the differences achieved statistical significance. It was therefore concluded that motor activity was unaffected by treatment.

Brain weights:

Absolute brain weights were not adversely affected by treatment.

Anatomical measurements:

The anatomical measurements made of the cerebral hemispheres did not indicate any differences between the controls and treated animals.

Macroscopic pathology:

Macroscopic examination of animals killed on completion of the treatment period revealed no treatment-related findings.

Microscopic pathology:

There were no changes in the tissues presented for neuropathological examination which were considered to be related to treatment with fluopicolide.

Findings considered related to treatment were seen in the fluopicolide target tissues liver and kidneys. An increased incidence of centrilobular hepatocytic hypertrophy of the liver, when compared with the controls, was observed in males and females given 10,000 ppm and in males given 1,400 ppm. Centrilobular hepatocytic hypertrophy is a common finding in the livers of rodents which have been administered xenobiotics and, as such, is considered to be an adaptive change of no toxicological significance.

In addition, an increased incidence and/or severity of hyaline droplets in the cortical tubules was observed in the kidneys of males given 1,400 or 10,000 ppm. This was associated with increased incidences and severities of other degenerative or regenerative changes in the kidneys including inflammation, casts and dilatation. Hyaline droplets have been recognized as indicator of changes associated with the accumulation of α2µ-globulin in the cortical tubules of affected kidneys. Since the α2µ-globulin is an adult male rat-specific protein it is widely accepted that the renal effects induced in male rats by chemicals causing α2µ-globulin accumulation are unlikely to occur in humans[34].
The histopathological changes due to treatment are summarized in Table 5.7.1- 18.

[34] Hard GC, Rodgers IS, Baetcke KP, Richards WL, McGaughy RE, Valcovic LR. Hazard evaluation of chemicals that cause accumulation of alpha 2u-globulin, hyaline droplet nephropathy, and tubule neoplasia in the kidneys of male rats. Environ Health Perspect. 1993 Mar;99:313-49

Table 5.7.1- 18: Histopathological changes related to treatment

Findings	Score	Dose level (ppm)							
		Males				Females			
		0	200	1,400	10,000	0	200	1,400	10,000
Liver									
Number examined	-	10	10	10	10	10	10	10	10
Centrilobular hepatocytic hypertrophy	Slight	0	0	9	10	0	0	0	6
	Total	0	0	9***	10***	0	0	0	6**
Kidney									
Number examined	-	10	10	10	10	10	10	10	10
Cortical tubules with hyaline droplets	Minimal	3	3	0	0	0	0	0	0
	Slight	2	1	8	0	0	0	0	0
	Moderate	0	1	1	10	0	0	0	0
	Total	5	5	10*	10*	0	0	0	0
Interstitial inflammation	Minimal	2	2	3	3	0	0	1	0
	Slight	0	0	2	4	0	0	0	0
	Moderate	0	0	0	2	0	0	0	0
	Total	2	2	5	9**	0	0	1	0
Granular casts-medulla	Minimal	0	0	2	2	0	0	0	0
	Slight	0	0	0	3	0	0	0	0
	Moderate	0	0	0	5	0	0	0	0
	Total	0	0	2	10***	0	0	0	0
Cortical tubular dilatation	Present	0	0	0	7**	0	0	0	0
Hyaline tubular casts	Minimal	0	3	3	2	0	0	0	0
	Slight	0	0	0	2	1	0	0	0
	Total	0	3	3	4	1	0	0	0

* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, statistically different to control

III. Conclusion

There was no evidence of neurotoxicity following the continuous administration of fluopicolide to CD rats for 13 weeks at dietary concentrations up to 10,000 ppm. Neurobehavioral screening and macroscopic and histopathological examination of the associated tissues (including anatomical measurements of the brain) revealed no treatment-related findings. In addition, no unusual signs or patterns of behaviour were observed at all routine observations.

It is concluded that the administration of fluopicolide to CD rats for 13 weeks via the diet at concentrations of up to 10,000 ppm (equivalent to 781 mg/kg bw/day in males and 866 mg/kg bw/day in females) did not result in any neurotoxicity. Thus, the NOAEL for neurotoxicity was >10,000 ppm (equivalent to 781 mg/kg bw/day in males and 866 mg/kg bw/day in females). Adaptive changes occurred in the liver at 1,400 and 10,000 ppm and male rat-specific 'hydrocarbon nephropathy' occurred at 1,400 and 10,000 ppm. Therefore, the NOAEL for systemic toxicity in the 13-week neurotoxicity study was 200 ppm (15.0 mg/kg bw/day in males and 18.0 mg/kg bw/day in females) based on impaired growth and histopathological changes in kidney of male animals at dose levels of ≥1,400 ppm (equivalent to 106.6 mg/kg bw/day in males and 125.2 mg/kg bw/day in females).

Assessment and conclusion by applicant:

An acceptable study yielding valid conclusions.

CA 5.7.2 Delayed polyneuropathy studies

Fluopicolide is not an organophosphate compound, therefore a delayed polyneuropathy is not an expected effect, so that respective studies on such a potential were not necessary and not conducted.