WHO FOOD ADDITIVES SERIES: 79

Prepared by the eighty-eighth meeting of the
Joint FAO/WHO Expert Committee
on Food Additives (JECFA)

Toxicological evaluation of certain veterinary drug residues in food

Food and Agriculture Organization of the United Nations

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World Health Organization, Geneva, 2021

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PREFACE

The monographs contained in this volume were prepared at the eighty-eighth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), which met at the headquarters of the Food and Agriculture Organization of the United Nations (FAO) in Rome on 22–31 October 2019. These monographs summarize the data on the safety of residues in food of selected veterinary drugs reviewed by the Committee.

The eight-eighth report of JECFA has been published by WHO as WHO Technical Report No. 1023. Reports and other documents resulting from previous meetings of JECFA are listed in Annex 1. The participants in the meeting are listed in Annex 3 of the present publication. A summary of the conclusions of the Committee is given in Annex 4.

JECFA serves as a scientific advisory body to FAO, WHO, their Member States and the Codex Alimentarius Commission, primarily through the Codex Committee on Food Additives, the Codex Committee on Contaminants in Food and the Codex Committee on Residues of Veterinary Drugs in Foods, regarding the safety of food additives, residues of veterinary drugs, naturally occurring toxicants and contaminants in food. Committees accomplish this task by preparing reports of their meetings and publishing specifications or residue monographs and toxicological monographs, such as those contained in this volume, on substances that they have considered.

The toxicological monographs contained in this volume are based on working papers that were prepared by WHO experts. A special acknowledgement is given at the beginning of each monograph to those who prepared these working papers. The monographs were edited by Dr Hilary Cadman, Bellingen, Australia. Toxicological monographs were not prepared for all of the substances listed in Annex 4.

Many unpublished proprietary reports are submitted to the Committee by various producers of the veterinary drugs under review and in many cases represent the only data available on those substances. The WHO experts based the working papers they wrote on all the data that were submitted, and all these reports were available to the Committee when it made its evaluations.

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Any comments or new information on the biological or toxicological properties of the compounds evaluated in this publication should be addressed to: Joint WHO Secretary of the Joint FAO/WHO Expert Committee on Food Additives, Department of Food Safety and Zoonoses, World Health Organization, 20 Avenue Appia, 1211 Geneva 27, Switzerland.

RESIDUES OF VETERINARY DRUGS

Diflubenzuron (addendum)

First draft prepared by

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1. Explanation

Diflubenzuron (International Union of Pure and Applied Chemistry [IUPAC] name: 1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl)urea; Chemical Abstracts Service [CAS] No. 35367–38–5) is an acyl urea derivative (halogenated benzoylphenylurea).

Diflubenzuron is approved for use as a veterinary drug in two Member States for the treatment of sea lice (*Lepeophtheirus salmonis* and *Caligus rogercresseyi*) infestations in Atlantic salmon (*Salmo salar*) at an oral dose of 3–6 mg/kg body weight (bw) in feed for 14 consecutive days, with a withdrawal period in the range 105–300 degree days. It is also used as an insecticide or acaricide in agriculture and forestry against larvae of Lepidoptera, Coleoptera, Diptera and Hymenoptera, and as a vector control agent in drinking-water sources and drinking-water storage containers.

The mechanism of action of diflubenzuron is to inhibit the formation of new chitin in the insect cuticle during the moulting process, by inducing both chitinase and phenoloxidase.

Diflubenzuron was previously evaluated at the 81st Joint Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA) meeting *(1)*. At that meeting, the Committee was unable to establish an acceptable daily intake (ADI) for diflubenzuron, because it could not be assured that there would be an adequate margin of safety from diflubenzuron's use as a veterinary drug in the absence of adequate information on exposure to 4-chloroaniline ([CAS No. 106–47–8]; *p*-chloroaniline; *p*-chlorobenzenamine) – a potentially genotoxic and carcinogenic metabolite and/or degradate of diflubenzuron – and on whether, and to what extent, diflubenzuron can be metabolized to 4-chloroaniline in humans.

The Committee also concluded that it was not possible to recommend maximum residue limits (MRLs) for diflubenzuron, and requested the following additional information to assist in a further evaluation:

- a comparative metabolism study of diflubenzuron in humans and rats (e.g. in hepatocytes);
- information on 4-chloroaniline exposure associated with the consumption of treated fish;
- information on the amount of 4-chloroaniline (if present) as an impurity in the product formulation;
- information on the amount of 4-chloroaniline generated during food processing; and
- a method suitable for monitoring diflubenzuron residues in fish muscle and fillet (muscle+skin in natural proportions).

The Committee noted that the toxicity of diflubenzuron has been previously evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) in 1981, 1985 and 2001 *(2-4)*, and by the WHO Task Group on Environmental Health Criteria for Diflubenzuron in 1996 *(5)*. In 2001, JMPR established an ADI of 0–0.02 mg/kg bw for diflubenzuron, based on the no-observed-adverse-effect level (NOAEL) of 2 mg/kg bw per day for haematological effects observed in 2-year toxicity studies in rats and a 52-week toxicity study in dogs. JMPR in 2019 was unable to address concerns raised about 4-chloroaniline arising from the use of diflubenzuron, owing to the lack of data, but noted that diflubenzuron was on the agenda of the 88th JECFA meeting. The Committee noted that the toxicity of 4-chloroaniline has been previously evaluated by the International Programme on Chemical Safety (IPCS) *(6)*.

Dietary exposure to diflubenzuron residues may occur through its use as a veterinary drug and as a pesticide. However, exposure to the diflubenzuron metabolite and contaminant 4-chloroaniline may potentially occur through several routes:

- 4-chloroaniline may be a contaminant of diflubenzuron formulations applied as a veterinary drug or as a pesticide;
- diflubenzuron residues may be metabolized to 4-chloroaniline in humans;
- diflubenzuron residues may be metabolized to 4-chloroaniline in animals and plants, which in turn may be a food source for humans and other animals;
- high-temperature processing of foods containing diflubenzuron residues from veterinary drug use or pesticide application may result in production of 4-chloroaniline; and
- exposure to 4-chloroaniline may occur from consumer products such as dyed or printed textiles and papers, biocides, cosmetics and pharmaceutical products.

Consequently, in its assessment the Committee considered all the routes of exposure to diflubenzuron as well as 4-chloroaniline.

The present evaluation was conducted at the request of the Codex Committee on Residues of Veterinary Drugs in Foods (CCRVDF), as a follow-up.

No additional information was submitted by the sponsor for the toxicological and microbiological evaluation of 4-chloroaniline. The Committee conducted a comprehensive literature search covering the period 1981–2019 using the following databases: PubMed, Web of Science, Science Direct, Food Science and Technology Abstracts, Scopus, OneFile (GALE), AGRIS, Wiley Online Library, Taylor and Francis Online, and SpringerLink. The keywords "diflubenzuron", "Atlantic salmon", "salmon", "metabolism", "comparative metabolism", "4-chloroaniline", "food processing", "cooking" and "exposure" with the Boolean operators (AND and OR) were used.

This formal literature search did not identify any articles relevant to the current evaluation. However, an open literature search identified a few papers on 4-chloroaniline relevant to the assessment *(7-10)*. Most of the studies were not conducted according to good laboratory practice (GLP), but were of sufficient quality to be included in this evaluation.

The Committee considered the information previously evaluated by JECFA in 2015 *(1)* and confirmed the conclusions reached at that meeting on study interpretation. Only information relevant to this follow-up evaluation of diflubenzuron and 4-chloroaniline is included here.

2. Biological data

2.1 Biochemical aspects

No additional data on biochemical aspects were submitted by the sponsor, and no new publications on biochemical aspects not addressed at the 81st meeting were identified.

Diflubenzuron is rapidly absorbed to a moderate extent from the gastrointestinal tract. In a single-dose oral study with ^{14}C-labelled diflubenzuron in rats, about 30% of the administered dose was absorbed at 5 mg/kg bw, and less was absorbed at 100 mg/kg bw. Once absorbed, diflubenzuron is extensively metabolized and rapidly excreted, mostly in the urine, although some enterohepatic circulation occurs. In the radiolabel study, more than 90% of the administered dose (5 and 100 mg/kg bw) was excreted within 24 hours. When mice were given a single oral dose of diflubenzuron at 12, 64, 200 or 920 mg/kg bw, excretion was almost complete within 48 hours *(1)*.

The primary metabolic pathways are hydroxylation of the aniline ring, cleavage of the ureido bridge and conjugation, mainly with sulfate. In rats, about 80% of the metabolites were identified as involving hydroxylation of the phenyl moieties of diflubenzuron, and approximately 20% underwent scission at the ureido bridge *(1)*.

4-Chloroaniline was not detected in bile or urine using a method with a limit of quantification (LOQ) of 7.5 ng/mL in rats. A radiolabel study with rats given a single dose of [U-14C-anilino]diflubenzuron at 104 mg/kg bw also did not identify 4-chloroaniline in urine. 4-Chloroaniline was not detected (limits of detection [LODs] not given) in urine or faeces of sheep or cow following administration of a single oral dose of diflubenzuron at 10 mg/kg bw, or in rat urine following administration of a single oral dose of diflubenzuron at 5 mg/kg bw. However, when diflubenzuron was given as a single oral dose of 5 mg/kg bw, 4-chloroaniline was detected in small quantities in swine urine (1.03% of the oral dose) and chicken excreta (0.44% of the dose) *(1)*.

When diflubenzuron was given as a single oral dose, 4-chlorophenylurea (CPU), a metabolite that may be reduced to 4-chloroaniline, was detected in small quantities in the urine of swine (0.82% of a 5 mg/kg bw dose), in the urine of cow (0.6% of a 10 mg/kg bw dose) and in chicken excreta (3.14% of a dose of 5 mg/kg bw) *(1)*.

Publicly available reports indicate that in sheep, swine and chicken, 4-chloroaniline has been found as a minor metabolite. In cattle, no or very low levels have been found *(11)*.

Table 1
Metabolites of diflubenzuron detected in different species

Species	Strain	Sex	Hours	Percentage					Detected 4-chloroaniline (ng/mL)
				4-Chloroaniline	N-(4-chloro-phenyl)-acetamide	4-Chloro-phenylurea	A more polar metabolite	5-Chloro-2-aminophenol	
Rat	Fischer F-344	Male	3	–	11	–	–	–	9
			24	1	32	1	2	1	23
Pig	Landrace – Large White	Male	3	3	19	–	–	–	38
			24	2	69	2	–	–	23
Goat	Alpine	Male	3	4	3	–	–	–	43
			24	–	4	–	–	–	6
Human	Not applicable	Male	3	–	–	–	–	–	7
			24	–	1–2	1–2	–	–	3

A comparative in vitro metabolism assay was conducted using rat, pig, goat and human hepatocytes *(12)*. Following incubation of ^{14}C-diflubenzuron diflubenzuron (10 μM) for 3 and 24 hours with cells, diflubenzuron is most readily metabolized by hepatocytes from pigs, followed by rats, goats and humans, with less formation of 4-chloroaniline in humans than in rats (Table 1).

The metabolites were N-(4-chlorophenyl)acetamide (acetylated metabolite of 4-chloroaniline), which accounted for 19% of the sample radioactivity in pig and 3% in goat; and 4-chloroaniline, which accounted for 3% in pig and 4% in goat. In the rat, only the metabolite acetylated 4-chloroaniline was detected, in 11%. After incubating human hepatocytes for 3 hours, there was no evidence of metabolism using radiochemical detection techniques.

2.2 Toxicological studies

2.2.1 Diflubenzuron

No additional data on toxicity were submitted by the sponsor. This section summarizes the information from the monograph developed for the 81st meeting.

(a) Acute toxicity

Diflubenzuron was of low acute toxicity when given to mice and rats by the oral, inhalation or dermal route. The oral median lethal dose (LD_{50}) was greater than 4600 mg/kg bw in mice and rats, the dermal LD_{50} was greater than 10 000 mg/

kg bw in rats, and the inhalation median lethal concentration (LC_{50}) was greater than 2.9 mg/L in rats *(1)*.

Diflubenzuron was not irritating to the skin of rabbits and was slightly irritating to the eyes of rabbits. Diflubenzuron was not a skin sensitizer in a study in guinea-pigs *(1)*.

The primary target for toxicity is the erythrocytes, with secondary effects on liver and spleen. Dose-related methaemoglobinaemia has been consistently demonstrated in both sexes of various species (mice, rats and dogs) after short-term or long-term oral exposure to diflubenzuron *(1)*.

(b) Short-term studies of toxicity

In a 13-week study, rats were fed diets containing diflubenzuron at a concentration of 0, 160, 400, 2000, 10 000 or 50 000 mg/kg feed (equal to 0, 8, 20, 100, 500 and 2500 mg/kg bw per day, respectively). A range of dose-related changes in erythrocyte parameters (erythrocyte counts, haemoglobin, reticulocytes, methaemoglobin and sulfhaemoglobin) were noted in both sexes at 400 mg/kg feed and above, with minimal effects at 160 mg/kg feed. The absolute and relative weights of the spleen were increased in males at 160 mg/kg feed and above, and in females at 400 mg/kg feed and above for 7 weeks. Pathological findings included chronic hepatitis, haemosiderosis and congestion of the spleen, and erythroid hyperplasia of the bone marrow in all treated groups, and haemosiderosis in the liver at 400 mg/kg feed and above. A NOAEL could not be identified because there were small but statistically significant increases in methaemoglobin concentration, and associated changes in the spleen and bone marrow, at the lowest dose tested (160 mg/kg feed, equal to 8 mg/kg bw per day) *(1)*.

In a 13-week non-GLP-compliant study in dogs, animals received diets containing diflubenzuron at a concentration of 0, 10, 20, 40 or 160 mg/kg feed (equal to 0, 0.4, 0.8, 1.6 and 6.4 mg/kg bw per day, respectively). At week 6, haemoglobin concentration and erythrocyte count were reduced and methaemoglobin and free haemoglobin concentrations were increased at 160 mg/kg feed. At week 12, there was an increase in the myeloid:erythroid ratio in bone marrow at 160 mg/kg feed. A NOAEL of 40 mg/kg feed (equal to 1.6 mg/kg bw per day) was identified, based on changes in haematological end-points and bone marrow at 160 mg/kg feed (equal to 6.4 mg/kg bw per day) *(1)*.

(c) Long-term studies of toxicity

In a 52-week non-GLP-compliant study, dogs received gelatine capsules containing diflubenzuron at a dose of 0, 2, 10, 50 or 250 mg/kg bw per day. Various effects related to impaired erythrocytes were seen at the two highest doses from week 13 onwards. Increases in methaemoglobin and sulfhaemoglobin

concentrations and in platelet counts were seen at 10 mg/kg bw per day and above. The only histopathological findings were in the liver (increased pigmentation of Kupffer cells and macrophages) at 10 mg/kg bw per day and above. A NOAEL of 2 mg/kg bw per day was identified, based on effects on methaemoglobin and sulfhaemoglobin concentrations, platelet counts and hepatic pigmentation at 10 mg/kg bw per day *(1)*.

(d) Long-term studies of toxicity and carcinogenicity

In a chronic toxicity and carcinogenicity study, diflubenzuron was given to mice in the diet at a concentration of 0, 16, 80, 400, 2000 or 10 000 mg/kg feed (equal to 0, 1.2, 6.4, 32, 160 and 840 mg/kg bw per day for males and 0, 1.4, 7.3, 35, 190 and 960 mg/kg bw per day for females, respectively) for 91 weeks. Significant, dose-related changes were seen in a number of haematological parameters from week 26 onwards (methaemoglobin and sulfhaemoglobin at 80 mg/kg feed and above; haemoglobin at 2000 mg/kg feed and above; and leukocyte and erythrocyte counts at 10 000 mg/kg feed). At week 26, absolute spleen weights were significantly increased at 2000 mg/kg feed and above. Increased incidences of splenic siderocytes at 400 mg/kg feed and above and of pigmented Kupffer cells at 10 000 mg/kg feed were noted. A NOAEL of 16 mg/kg feed (equal to 1.2 mg/kg bw per day) was identified, based on methaemoglobin formation at 80 mg/kg feed (equal to 6.4 mg/kg bw per day). There was no evidence of carcinogenicity in this study *(1)*.

In a non-GLP-compliant chronic toxicity and carcinogenicity study in rats, animals received diflubenzuron in the diet at a concentration of 0, 10, 20, 40 or 160 mg/kg feed (equivalent to 0, 0.5, 1, 2 and 8 mg/kg bw per day, respectively) for 2 years. The achieved dietary concentrations and homogeneity of diflubenzuron in the feed were not confirmed. The NOAEL was 40 mg/kg feed (equal to 2 mg/kg bw per day), based on increases in methaemoglobin concentration and reduced free haemoglobin concentration at 160 mg/kg feed (equal to 8 mg/kg bw per day). There was no increase in the incidence of tumours in treated animals. However, the poor survival (<30% in all groups at termination) and limited range of tissues examined limited the power of this study to detect any carcinogenicity of diflubenzuron *(1)*.

In a GLP-compliant combined 2-year toxicity and carcinogenicity study, rats received diflubenzuron in the diet at a concentration of 0, 160, 620, 2500 or 10 000 mg/kg feed (equal to 0, 7.1, 28, 112 and 472 mg/kg bw per day for males and 0, 9.3, 37, 128 and 612 mg/kg bw per day for females, respectively). Erythrocyte parameters (e.g. methaemoglobin and sulfhaemoglobin concentrations) were altered, with no marked progression with duration and dosing. The main treatment-related histopathological findings were pigmented macrophages in the

spleen and liver and erythroid hyperplasia of the bone marrow at 620 mg/kg feed and above. A NOAEL for toxicity could not be identified, owing to increases in methaemoglobin and sulfhaemoglobin concentrations noted at 160 mg/kg feed (equal to 7.1 mg/kg bw per day), the lowest dose tested *(1)*.

The overall incidences of tumours were low, with no treatment- or dose-related findings. The overall NOAEL for toxicity in the 2-year studies in rats was 2 mg/kg bw per day, and the overall lowest-observed-adverse-effect level (LOAEL) was 7.1 mg/kg bw per day *(1)*.

(e) Long-term studies of genotoxicity

The genotoxicity of diflubenzuron was evaluated in an adequate range of assays, both in vitro and in vivo. No evidence of genotoxicity was found, other than two recent in vivo studies in which positive findings in micronucleus induction and comet formation in the peripheral blood (the target of toxicity) were reported in mice given diflubenzuron at a dose of 0.3, 1 or 3 mg/kg bw. The genotoxicity potency reported in this study was inconsistent with what was reported in other studies and has not been replicated. The Committee concluded that diflubenzuron is not genotoxic based on the weight of evidence of genotoxicity information available *(1)*.

In view of the lack of genotoxicity and the absence of carcinogenicity in mice and rats, the Committee concluded that diflubenzuron is unlikely to pose a carcinogenic risk to humans from the diet *(1)*.

(f) Reproductive and developmental toxicity

In a two-generation reproductive toxicity study, rats received diets containing diflubenzuron at a concentration of 0, 500, 5000 or 50 000 mg/kg feed (equal to 0, 42, 430 and 4300 mg/kg bw per day for males and 0, 36, 360 and 3800 mg/kg bw per day for females, respectively). Haematological parameters and the spleen were not examined in young animals. Reproductive parameters were not affected. Pup weights were reduced in a dose-related manner in the F1 generation, but not in the F2 generation. Alterations in erythrocyte parameters and increases in lymphocyte counts and platelet numbers were seen in all parental groups. The spleen was the primary target organ, showing increases in weight, congestion and haemosiderosis at all doses and an increase in the incidence of congested red pulp in F0 animals at the middle and high doses. Effects on liver included increased incidences of centrilobular hepatocyte hypertrophy at the middle and high doses and brown pigmentation of Kupffer cells in all treated groups. The NOAEL for reproductive effects was 50 000 mg/kg feed (equal to 3800 mg/kg bw per day), the highest dose tested. The NOAEL for offspring toxicity was 5000 mg/kg feed (equal to 360 mg/kg bw per day), based on reductions in pup

body weight at 50 000 mg/kg feed (equal to 3800 mg/kg bw per day) in the F1 generation. A NOAEL for parental toxicity could not be identified because of the haematological effects observed at all doses tested *(1)*.

In a developmental toxicity study, rats were dosed orally by gavage with diflubenzuron at 0 or 1000 mg/kg bw per day (the limit dose) from days 6 to 15 of gestation. The dams were killed on day 20 of gestation. No treatment-related effects on the dams or fetuses were noted. The NOAEL for both maternal and embryo/fetal toxicity was 1000 mg/kg bw per day, the only dose tested *(1)*.

In another developmental toxicity study, rabbits were dosed orally by gavage with diflubenzuron at 0 or 1000 mg/kg bw per day (the limit dose) from days 7 to 19 of gestation. The does were killed on day 28 of gestation. No treatment-related effects on the does or fetuses were noted. There was no evidence of developmental toxicity in rabbits. The NOAEL for both maternal and embryo/fetal toxicity was 1000 mg/kg bw per day, the only dose tested *(1)*.

2.2.2 4-Chloroaniline

(a) Short-term and long-term studies of toxicity

Repeated exposure to 4-chloroaniline leads to cyanosis and methaemoglobinaemia, followed by effects in blood, liver, spleen and kidneys, as evidenced by changes in haematological parameters, splenomegaly and haemosiderosis (from moderate to heavy) in spleen, liver and kidney, partially accompanied by extramedullary haematopoiesis. The LOAELs for a significant increase in methaemoglobin levels in rats and mice were 5 and 7.5 mg/kg bw per day, respectively, for a 13-week oral gavage administration of 4-chloroaniline. The LOAEL for a 103-week oral gavage study in rats (with administration 5 days/week) was 2 mg/kg bw per day, based on a significant increase in methaemoglobin levels and fibrotic changes of the spleen in male rats; hyperplasia of bone marrow was observed in female rats at and above 6 mg/kg bw per day. This information demonstrated that 4-chloroaniline exhibits toxicity end-points similar to those of diflubenzuron, but is more potent than diflubenzuron *(1)*.

(b) Long-term studies of toxicity and carcinogenicity

In a dietary carcinogenicity study in mice, animals received 4-chloroaniline at a concentration of 0, 2500 or 5000 mg/kg feed (equal to 0, 375 and 750 mg/kg bw per day, respectively) for 78 weeks, followed by a 13-week observation period. Decreased body weight gain was observed in treated animals. Non-neoplastic proliferative and chronic inflammatory lesions were found in the spleens of treated mice. There was an increased incidence of haemangiosarcomas in the spleen, liver, kidney and subcutaneous tissue (combined) for both sexes.

It was concluded that there was insufficient evidence to determine whether 4-chloroaniline was carcinogenic in mice *(1)*.

In a second carcinogenicity study in mice, animals were administered 4-chloroaniline by oral gavage in aqueous hydrochloric acid at 0, 3, 10 or 30 mg/kg bw per day, 5 days/week, for 103 weeks. Incidences of proliferation of haemopoietic cells in the liver were increased in dosed females. Multifocal renal tubular pigmentation (haemosiderin) was observed in high-dose females. There were increases in the incidences of hepatocellular carcinomas in males dosed at 10 and 30 mg/kg bw per day (3/50, 7/49, 11/50, 17/50), incidences of combined hepatocellular adenomas and carcinomas in all treated males (11/50, 21/49, 20/50, 21/50) and incidences of haemangiosarcomas of the liver and spleen (combined) in males at 30 mg/kg bw per day (4/50, 4/49, 1/50, 10/50). It was concluded that there was some evidence of carcinogenicity in male mice and no evidence in female mice *(1)*.

In a dietary carcinogenicity study in rats, animals received 4-chloroaniline at a concentration of 0, 250 or 500 mg/kg feed (equivalent to 0, 12.5 and 25 mg/kg bw per day, respectively) for 78 weeks, followed by a 24-week observation period. Mesenchymal tumours (fibroma, fibrosarcoma, haemangiosarcoma, osteosarcoma and sarcoma not otherwise specified) in the spleen were observed in males at the high dose and in females at both doses; no tumours were found in the controls. It was concluded that there was insufficient evidence to determine whether 4-chloroaniline was carcinogenic in rats *(1)*.

In a second carcinogenicity study in rats, animals were administered 4-chloroaniline by oral gavage in aqueous hydrochloric acid at 0, 2, 6 or 18 mg/kg bw per day, 5 days per week, for 103 weeks. Changes in haematological parameters (e.g. decreases in haemoglobin concentration, erythrocyte count and haematocrit) were noted at various time points. Non-neoplastic findings included bone marrow hyperplasia, hepatic haemosiderosis and splenic fibrosis. The incidence of uncommon sarcomas of the spleen in high-dose male rats was significantly higher than that in the vehicle controls (fibrosarcomas, osteosarcomas or haemangiosarcomas, combined: 0/49, 1/50, 3/50, 38/50); some of these tumours metastasized to one or more sites. One mid-dose female developed fibrosarcoma, and one high-dose female developed osteosarcoma; the controls showed zero incidence of either of these tumours. The incidence of adrenal phaeochromocytomas or malignant phaeochromocytomas combined was significantly higher in the high-dose males. There was a non-significant increase in the incidence of phaeochromocytomas in high-dose females (2/50, 3/50, 1/50, 6/50). It was concluded that there was clear evidence of carcinogenicity in male rats and equivocal evidence in female rats *(1)*.

The oral gavage carcinogenicity study is considered to be more appropriate than the dietary admixture feeding study for determining carcinogenicity because

4-chloroaniline is unstable in feed, and mice and rats in the feeding studies were dosed for 78 weeks, then killed and examined for histopathology following a further 13-week (mice) or 24-week (rats) observation period. Nonetheless, both studies showed some similar effects: splenic toxicity in male and female rats, a treatment-related increase in uncommon splenic sarcomas in male rats and a treatment-related increase in haemangiosarcomas in male mice *(1)*.

(c) Special study

No new publications on the toxicity of diflubenzuron or 4-chloroaniline that were not addressed at the 81st meeting were identified, other than a single article on the genotoxicity and a possible mode of action for the tumorigenicity of 4-chloroaniline *(10)*. The purpose of the article was to investigate a possible mode of action for splenic tumour formation using a transgenic rodent in vivo gene mutation assay in Big Blue® TgF344 rats. A parallel micronucleus analysis in peripheral blood was also performed in these rats. The derived strain of transgenic rats was the same strain used for a carcinogenicity study of 4-chloroaniline *(13)*.

Toxicity of metabolite – 4-chloroaniline

A 4-week oral toxicity study using TgF344 rats

A 4-week oral toxicity study of 4-chloroaniline was conducted using Big Blue® F344 rats – Taconic nomenclature: F344-TgN(lamda/lacI), abbreviated as TgF344. Aniline, a compound with similar toxicity to 4-chloroaniline, was also investigated in this study. A group of six male TgF344 rats was administered 4-chloroaniline (purity, 100%; batch no. SHBG6394V) by gavage at dose levels of 0.5, 15 and 60 mg/kg bw per day in 1% carboxymethylcellulose (CMC) in deionized water for 28 days. The dose of 60 mg/kg bw was reduced to 30 mg/kg bw on day 10 after starting, owing to serious toxicity. A group of six TgF344 male rats were also given aniline (purity, 100%; batch no. SHBG6394V) at a dose level of 100 mg/kg bw per day by gavage. Both compounds were purchased from Sigma-Aldrich (St. Louis, Missouri). Accuracy of concentration, homogeneity and stability of dosing formulations of 4-chloroaniline and aniline in 1% CMC in deionized water collected in week 1 and week 4 of dosing was confirmed by analysis by high-performance liquid chromatography (HPLC). This study was conducted in compliance with GLP and in accordance with Organisation for Economic Co-operation and Development (OECD) *Test guideline 488 (14)*. Although the original purpose of this study was to clarify transgenic rodent somatic and germ cell gene mutation of 4-chloroaniline, the study also examined observation and systemic toxicity parameters related to 4-chloroaniline or aniline. No histopathological examination was conducted for any tissues or organs.

Feed consumption was statistically significantly decreased in the first week at 60 mg/kg bw. The decrease was accompanied by clinical signs such as pallor and decreased motor activity. Hence, the high dose was reduced to 30 mg/kg bw from day 10. No further details are provided in the paper. Spleen weight was statistically significantly increased (by ~twofold) in rats treated with 4-chloroaniline at 15 mg/kg bw and above, and with aniline. Liver weight was significantly increased in the aniline treated group.

Major 4-chloroaniline-treatment-related changes in haematological parameters were decreased red blood cells, increased mean corpuscular volume (MCV) at 15 mg/kg bw and above, and lower haemoglobin, increased mean corpuscular haemoglobin concentration (MCHC) at 60/30 mg/kg bw. Decreased red blood cells, haemoglobin, haematocrit and MCHC, and increased MCV and MCHC, were observed in the aniline treatment group. The absolute number of reticulocytes (ABRET) was markedly increased from 15 mg/kg bw per day of 4-chloroaniline and at 100 mg/kg bw per day of aniline. Similar haematological and other effects were also observed in 4-chloroaniline treated animals in the National Toxicology Program study *(13)*. Only limited changes in blood biochemical parameters were observed. The most notable change was a statistically significant increase in calcium in the 100 mg/kg aniline group, and decreases in cholesterol and albumin/globulin (A/G) ratio with 60/30 mg/kg 4-chloroaniline. Methaemoglobin was not detected in control rats or in those treated with 0.5 mg/kg 4-chloroaniline. Methaemoglobin values at doses of 15 and 60/30 mg/kg of 4-chloroaniline, and 100 mg/kg of aniline were statistically significantly increased above control values, to 3.87 ±1.4% (mean ±standard deviation), 8.65 ±1.51%, and 7.3 ±0.99%, respectively. These increases in methaemoglobin formation confirm the findings reported in previous rodent studies with 4-chloroaniline *(13)* and aniline *(15)*.

Major changes for systemic toxicity are summarized in Table 2.

The results indicated that repeat oral exposure to tumorigenic dose levels of both compounds leads to cyanosis and methaemoglobinaemia, followed by effects in blood and spleen, manifested as changes in haematological parameters and splenomegaly. Exposure to 4-chloroaniline and aniline at doses similar to those that cause carcinogenicity following chronic exposure (12.7 and 72 mg/kg, respectively) leads to haemolytic anaemia. Treatment with 4-chloroaniline at 15 mg/kg bw and above and with aniline at 100 mg/kg bw per day resulted in statistically significant decreases in red blood cells and haemoglobin, and statistically significant increases in methaemoglobin, MCV, MCH and ABRET when compared with the control. This demonstrates that following exposure to either 4-chloroaniline or aniline, and due to the subsequent formation of methaemoglobin, exposed rats responded by increasing their production of red blood cells with altered physiology to address their increase oxygen requirements *(10)*.

Table 2
Summary of systemic toxicity observed in TgF344 rats treated with 4-chloroaniline and aniline

		4-Chloroaniline (mg/kg bw per day)			Aniline (mg/kg bw per day)
Parameters	**Vehicle control**	**0.5**	**15**	**60/30**	**100**
No. of rats examined	6	6	6	6	6
Spleen weight					
Increased % to control value	0	–[a]	78.5*	136.9	117.5
Haematology					
RBC (M/μL)	9.033 ±0.254	9.204 ±0.528	7.538 ±0.393*	6.547 ±0.388*	6.513 ±0.314*
HGB (g/dL)	15.03 ±0.44	15.10 ±0.70	14.12 ±0.71	13.85 ±0.67*	12.93 ±0.44*
HCT (%)	50.93 ±1.30	51.54 ±2.93	48.30 ±2.35	47.60 ±2.91	44.85 ±1.93*
MCV (fL)	56.40 ±0.65	56.04 ±0.59	64.14 ±1.49*	72.70 ±0.77*	68.90 ±10.1*
MCH (pg)	16.65 ±0.37	16.44 +0.36	18.72 ±0.39*	21.17 ±0.33*	19.85 ±0.48*
MCHC (g/dL)	29.50 ±0.43	29.30 ±0.47	29.20 ±0.12	29.12 ±0.42	28.82 ±0.39*
ABRET (×109/L)	278.23 ±31.84	295.18 ±15.14	592.12 ±70.95*	816.68 ±53.88*	775.88 ±63.44*
MetHb formation					
	nd	nd	3.87 ±1.4%	8.65 ±1.51%	7.3 ±0.99%

ABRET: absolute reticulocytes; bw: body weight; HCT: haematocrit; HGB: haemoglobin; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration; MCV: mean corpuscular volume; MetHb: methaemoglobin; nd: none detectable; RBC: red blood cell.
* Statistically significant from control value at $P<0.05$ by Dunnett's test.
[a] No specific data provided, but description of no statistical significance.
Source: Koenig et al. (2018) *(10)*.

Micronucleus assay in vivo and mutation frequency using TgF344 rats

This study was the same as the 4-week oral toxicity study in TgF344 male rats described above. Peripheral blood was taken on days 4 and 29 for analysis of micronucleated reticulocytes (MnRETs). About 200 μL of blood was collected and subsequently processed and analysed. Up to 20 000 reticulocytes per animal were analysed.

All animals necropsied on test day 31 were used for transgene mutant frequency quantification. Spleen and bone marrow, major target organs of 4-chloroaniline or aniline, and liver were selected for the analysis. TgF344 male rats were exposed by oral gavage to a 20 mg/kg dose of N-ethyl-N-nistrosourea (ENU) on days 1, 2, 3, 12, 19 and 26 as a positive control. Isolated DNA was processed using Packaging Reaction Mix (New York University, New York, New York), following methods described by Agilent *(16-18)*. Packaged phage was evaluated for overall phage titre and mutant phage titre, to permit calculation of a mutant frequency for each tissue, based on methods described by Agilent *(17)*. Packaged phage was incubated overnight at 37 ±1.0 °C, then scored for plaque formation and titre determination; cII mutant selection plates were incubated at

Table 3
Micronucleus assay – summary of TgF344 rats treated with 4-chloroaniline or aniline

		4-Chloroaniline (mg/kg bw per day)			Aniline (mg/kg bw per day)
Parameters	**Vehicle control**	**0.5**	**15**	**60/30**	**100**
No. of rats examined	6	6	6	6	6
On day 4					
% RET mean ±SD (range)	1.2 ±0.5 (0.77–2.03)	1.3 ±0.4 (0.62–1.70)	2.5 ±1.0 (0.66–3.28)	6.4 ±1.4** (5.41–8.16)	2.8 ±1.1** (1.30–4.27)
% RET change from control	–	8	108	433	133
% MnRET mean ±SD (range)	0.04 ±0.01 (0.017–0.054)	0.03 ±0.01 (0.015–0.053)	0.14 ±0.03** (0.094–0.175)	0.46 ±0.03** (0.436–0.545)	0.11 ±0.03** (0.073–0.157)
Total cell score					
MnRET	**43**	**34**	**167**	**619**	**130**
RET	**113 270**	**112 994**	**120 516**	**135 649**	**120 769**
On day 29					
% RET mean ±SD (range)	0.6 ±0.2 (0.21–0.81)	1.0 ±0.2** (0.71–1.13)	3.5 ±0.7** (2.72–4.60)	5.9 ±0.6** (4.88–6.40)	4.8 ±1.3** (2.80–6.65)
% RET change from control	–	67	483	883	700
% MnRET mean ±SD (range)	0.06 ±0.02 (0.035–0.089)	0.05 ±0.01 (0.031–0.070)	0.20 ±0.04** (0.154–0.237)	0.21 ±0.04** (0.163–0.280)	0.22 ±0.06** (0.177–0.283)
Total cell score					
MnRET	**59**	**82**	**292**	**302**	**324**
RET	**110 080**	**156 752**	**145 825**	**140 470**	**145 516**

bw: body weight; MnRET: micronucleated reticulocyte; RET: reticulocyte; SD: standard deviation.
** Statistically significant from control value at $P < 0.01$ one-way ANOVA with post-hoc Dunnett's test.
Source: Koenig et al. (2018) *(10)*.

24 ±0.5 °C, then scored for mutant plaques after 40–48 hours of incubation. A total of at least 125 000 phages were obtained from a minimum of two packagings.

The result of the micronucleus assay are described in Table 3.

The increase in percentage reticulocytes in the micronucleus analysis indicates that both 4-chloroaniline and aniline had an effect on cell division in peripheral blood, possibly due to anaemia, since 4-chloroaniline treated animals usually experienced anaemia *(13)*. As a consequence of the increase in red blood cell generation, a significant increase in MnRETs was detected in peripheral blood on both test day 4 and test day 29 at 15 mg/kg bw per day and above with 4-chloroaniline, and at 100 mg/kg bw per day with aniline. The report considered that the correlation between reticulocyte induction and MnRETs for individual animals on day 4 was robust (R-squared value of 0.8875). However, on day 29, the level of MnRETs plateaued, possibly due to compensatory mechanisms, while the

Table 4
Big Blue® mutant frequency using TgF344 male rats treated with 4-chloroaniline or aniline

		4-Chloroaniline (mg/kg bw per day)			Aniline (mg/kg bw per day)
Parameters	**Vehicle control**	**0.5**	**15**	**60/30**	**100**
No. of rats examined	6	6	6	6	6
On day 29	**Mutant frequency mean ±SD (×10–6)**				
Liver	47.3 ±21.9	36.9 ±7.2	33.6 ±15.8	43.2 ±20.3[a] 84.9 ±82.8[b]	32.8 ±2.5
Spleen	23.6 ±5.8	33.5 ±13.5	24.1 ±10.8	23.2 ±14.0	22.8 ±7.8
Bone marrow	19.3 ±10.7	18.1 ±1.9	22.1 ±7.3	16.6 ±12.7	16.8 ±9.1

bw: body weight; SD: standard deviation.
[a] Result including replacement animal (original animal excluded).
[b] Result including original animal (replacement animal 3524 excluded).
Source: Koenig et al. (2018) *(10)*.

induction of reticulocytes at the affected dose levels was greater than that seen on day 4. The results are summarized in Table 4.

No increase in mutant frequency at the cII gene was observed in the spleen, bone marrow or liver of Big Blue® F344 rats following 28 days of repeated exposure to 4-chloroaniline or aniline. However, mutant frequencies in the ENU-treated positive control animals were statistically significantly increased over the mutant frequencies in control amimals for liver ($P = 0.007$), spleen ($P < 0.001$) and bone marrow ($P = 0.009$).

Other in vitro assays

Information on other 4-chloroaniline genotoxicity tests was integrated into the tables *(7-9, 12)*.

2.3 Observations in humans

The sponsor did not submit any newly available data.

3. Comments

3.1 Biochemical data

In a study not reported in the previous evaluation, the comparative metabolism of diflubenzuron was investigated in vitro using rat, pig, goat and human hepatocytes

(12). Following incubation of ^{14}C-diflubenzuron (10 μM) for 3 and 24 hours, diflubenzuron was most readily metabolized by hepatocytes from pigs, followed by those from rats, goats and humans, with less formation of 4-chloroaniline by human hepatocytes than by rat hepatocytes. The Committee therefore concluded that health effects of possible production of 4-chloroaniline in human consumers upon exposure to diflubenzuron as a residue in food are adequately covered by studies of the toxicity of diflubenzuron in rats.

3.2 Toxicological data

4-Chloroaniline has been investigated in a wide range of in vitro and in vivo genotoxicity studies of varying quality. It was largely negative in bacterial mutation assays, but occasional positive results were observed with metabolic activation. Positive results, with and without metabolic activation, have been reported in mammalian cell gene mutation assays in vitro, and in clastogenicity studies in vitro and in vivo. 4-Chloroaniline was negative in the rat unscheduled DNA synthesis test in vivo and in a newly evaluated ToxTracker® assay in vivo; both of these tests would reflect DNA-reactive genotoxicity. 4-Chloroaniline showed no evidence of inducing DNA damage in the spleen in vivo, as assessed by a newly evaluated comet assay; effects in the liver were observed only at hepatotoxic doses and were considered secondary to cytotoxicity.

In a published study conducted according to GLP and not previously evaluated, repeat gavage exposure of Big Blue® F344 rats to 4-chloroaniline for 28 days did not produce any increase in cII transgene mutant frequency in the tissues analysed (i.e. spleen, liver and bone marrow). An increase in micronuclei was seen at both 4 and 29 days at a dose of 4-chloroaniline of 15 mg/kg bw and higher. At the same dose levels, significant reductions in red blood cell numbers, increases in the absolute numbers of reticulocytes and increased levels of methaemoglobin were observed.

The Committee concluded that 4-chloroaniline is clastogenic in vitro and in vivo, and mutagenic in vitro but not in vivo. The Committee considered that the genotoxicity of 4-chloroaniline was due to a mechanism secondary to reactive oxygen production rather than a direct reaction of 4-chloroaniline with DNA, and that the effect would exhibit a threshold.

4-Chloroaniline induces splenic tumours in F344 rats at doses that are toxic to both red blood cells and the spleen. The splenic tumours have been defined as fibromas, fibrosarcomas, osteosarcomas, haemangiosarcomas and sarcomas not otherwise specified. In B6C3F$_1$ mice, 4-chloroaniline appeared to increase the incidence of hepatic carcinomas while decreasing the incidence of adenomas. There was no change in the combined incidence of hepatic tumours with dose.

No mode of action is available for the tumorigenic response in mouse liver. However, hepatic tumours were observed only in male mice, not in female mice or in rats. Such tumours often occur through a threshold-dependent mode of action. A range of studies support a possible mode of action for the splenic tumours in rats, involving covalent modification of haemoglobin, accompanied by the formation of methaemoglobin, resulting in damaged erythrocytes, an increase in Heinz body formation and stimulation of erythropoiesis. The damaged erythrocytes are filtered by the spleen, which would lead to an increase in iron deposition, production of reactive oxygen species, protein oxidation and lipid peroxidation. These changes would result in a progression of pathological damage in the spleen, leading to tumours. The proposed mode of action is plausible; together with the absence of mutations in the spleen in the in vivo gene mutation study, it supports a threshold mode of action secondary to induction of significant red cell damage.

The Committee concluded that, based on the absence of gene mutations in vivo and the modes of action proposed, the carcinogenicity of 4-chloroaniline would exhibit a threshold.

The Committee concluded that the database on 4-chloroaniline was insufficient to enable the establishment of health-based guidance values for 4-chloroaniline, and therefore considered the application of the threshold of toxicological concern (TTC) approach for its risk characterization. This is based on the risk-based decision-tree approach for the safety evaluation of residues of veterinary drugs *(19)*, which was developed by the 70th meeting of JECFA *(20)* and subsequently revised by the 75th meeting *(21)*.

4. Evaluation

The Committee concluded that it was not necessary to establish an acute reference dose (ARfD) for diflubenzuron, in view of its low acute oral toxicity and the absence of developmental toxicity, and of any other toxicological effects likely to be elicited by a single dose.

The Committee established an ADI for diflubenzuron of 0–0.02 mg/kg bw, based on the NOAEL of 2 mg/kg bw per day for increased methaemoglobin and sulfhaemoglobin levels in a 2-year study of toxicity and carcinogenicity in rats, and for increased methaemoglobin and sulfhaemoglobin levels, platelet counts and hepatic pigmentation in a 1-year study of toxicity in dogs, applying a safety factor of 100. The LOAEL for effects seen in the 91-week study in mice was three times greater than the NOAEL on which the ADI is based.

As 4-chloroaniline does not exhibit DNA-reactive genotoxicity in vivo, its estimated chronic exposure can be compared with the TTC for a Cramer Class III compound; that is, 1.5 µg/kg bw per day. This would provide a margin of 8600 for the LOAEL value for splenic tumours in rats.

For the residue evaluation, the Committee considered a dataset submitted by the sponsor that provided data on the concentration of diflubenzuron and 4-chloroaniline residues in a depletion study with Atlantic salmon following the feeding of diflubenzuron in a commercial medicated feed. These data were submitted from the original sponsor that provided the studies that had been reviewed by the Committee at its 81st meeting. No additional data from other sources were received.

The Committee reviewed a field trial residue depletion study for diflubenzuron involving monitoring of both diflubenzuron and 4-chloroaniline in Atlantic salmon fillet over a period of 117 degree days postdose.

The analytical methods submitted by the sponsor to support the diflubenzuron residue depletion and the determination of 4-chloroaniline in salmon were also assessed. The study submitted by the sponsor was performed in compliance with GLP guidelines.

The Committee reconfirmed diflubenzuron as the marker residue (MR) and the MR to total radioactive residue (TRR) ratio of 0.9 established at its 81st meeting *(1)*.

4.1 Residue data – salmon

In the field trial study, Atlantic salmon weighing 3–4 kg held in seawater cages (average water temperature of 9 °C, minimum of 7.1 and maximum of 10.5 °C) received a daily dose of diflubenzuron (in the range 0.6–3.8 mg/kg bw) in medicated feed for 16 consecutive days. Twelve fish at each sampling time were selected randomly from the same cage at 0, 5.6, 9.7, 19.7, 48.7, 77.1 and 116.5 degree days, after the last administration of the medicated feed, corresponding to 14 hours and 1, 2, 5, 8 and 12 days postdose. The animals were slaughtered and fillet tissues collected. Diflubenzuron and 4-chloroaniline were determined in subsamples of the same fillet samples by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) and ultra-HPLC coupled to a high-resolution mass spectrometer (UHPLC-HRMS), respectively. The Committee did not receive the complete description of the analytical method for the determination of diflubenzuron in salmon fillet, including the sample preparation procedure and the full method validation report. The sponsor informed that the method had an LOQ of 10 µg/kg for diflubenzuron. The method used for the determination of

4-chloroaniline was fully described and validated. The limits of quantitation and detection were 1 and 0.33 µg/kg, respectively, for 4-chloroaniline.

The highest mean diflubenzuron concentration (4.16 mg/kg) was determined on day 1 (9.7 degree days) after the end of the treatment. The mean concentration declined to 0.20 mg/kg at 12 days (116.5 degree days). The highest concentrations of 4-chloroaniline, 1.27 µg/kg and 1.01 µg/kg, were determined in two fillet samples collected at 14 hours (5.6 degree days) and 1 day (9.7 degree days). Whereas the diflubenzuron mean concentrations ±standard deviation were 0.61 ±0.19 mg/kg and 0.20 ±0.11 mg/kg on days 8 and 12 post last dose, the concentrations of 4-chloroaniline were below the LOD (0.33 µg/kg) in all analysed samples from day 5 onwards.

The concentrations of diflubenzuron and 4-chloroaniline determined in the fillet of each animal (A1–A12) are shown in Table 5.

4.1.1 Analytical methods

The Committee assessed the validation data against the requirements for analytical methods, as published in the Codex guideline CAC-GL71-2009 *(22)*. The Committee reviewed the two methods submitted by the sponsor, one for the determination of diflubenzuron in salmon fillet and the other for the determination of 4-chloroaniline in salmon fillet.

Determination of diflubenzuron by LC-MS/MS: The Committee did not receive the complete description of the analytical method for the determination of diflubenzuron in salmon fillet, including the sample preparation procedure and the full method validation report, but the analysis was performed in a certified laboratory and the Committee considered it highly likely that the appropriate process was followed. In summary, water, acetonitrile and the internal standard are added to a 2.5 g salmon sample. The mixture is homogenized and extracted with petroleum ether. An aliquot of the water–acetonitrile phase is taken and reduced in volume. Further dilution is undertaken before quantitation of diflubenzuron by LC-MS/MS. The method LOQ was 10 µg/kg.

Determination of 4-chloroaniline by UHPLC-HRMS: In summary, the internal standard (^{13}C-4-chloroaniline), at a concentration of 10 µg/kg, is added to the homogenized salmon fillet. The analyte and internal standard are extracted by solvent extraction with a mixture of methanol: water: formic acid: sodium chloride 49.5:49.5:0.5:0.5 v/v/v/w. The extract is cleaned up by solid-phase extraction. The eluate is diluted with water and analysed by UHPLC-HRMS. The separation is carried out on a UHPLC C_{18} column under gradient elution. The mobile phase consists of water and methanol, containing 0.1% formic acid. The electrospray ionization source is operated in the positive ion mode. For the quantitation, an orbitrap mass spectrometer is used, operated in the parallel

Table 5

Concentrations of diflubenzuron and 4-chloroaniline in Atlantic salmon fillet following 16 days of daily administration (via feed) of diflubenzuron at a dose in the range 0.6–3.8 mg/kg bw

Time post dose (days)	Time post dose (degree days)	Concentration of the analyte: DFB (mg/kg) and PCA (µg/kg)											
		Analyte	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
0.6[a]	5.6	DFB	4.6	1.7	3.2	3.5	3.5	2.4	2.5	3.0	2.6	3.0	2.8	2.7
		PCA	1.27	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOD	<LOD	<LOQ	<LOQ
1	9.7	DFB	3.6	2.8	5.1	5.4	3.3	5.2	4.2	3.4	4.1	3.7	5.0	4.1
		PCA	<LOD	<LOD	<LOD	<LOD	<LOQ	<LOQ	<LOQ	1.01	<LOQ	<LOQ	<LOQ	<LOQ
2	19.7	DFB	2.6	1.6	1.9	0.9	1.2	3.6	1.6	2.5	1.7	2.0	1.4	3.6
		PCA	<LOQ	<LOD	<LOQ	<LOQ	<LOD	<LOQ	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOD
5	48.7	DFB	2.8	2.1	2.0	3.7	1.9	0.19	1.5	1.6	2.0	1.5	0.42	1.8
		PCA	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOQ	<LOD	<LOD
8	77.1	DFB	0.57	0.71	0.74	0.81	0.87	0.79	0.72	0.52	0.58	0.33	0.34	0.34
		PCA	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
12	116.5	DFB	0.041	0.19	0.29	0.4	0.23	0.11	0.4	0.17	0.14	0.17	0.17	0.11
		PCA	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD

A: animal; bw: body weight; DFB: diflubenzuron; LOD: limit of detection; LOQ: limit of quantification; PCA; 4-chloroaniline.

[a] 14 hours postdose. LOD: 0.33 µg/kg and LOQ: 1 µg/kg.

reaction monitoring mode. Two transitions are monitored, the first for the quantitation (mass-to-charge ratio [*m/z*] 128.0262 →93.0578) and the second (*m/z* 128.0262 →75.0237) for identity confirmation. The method was validated assessing the selectivity, linearity, intra-day and inter-day precisions, recovery, matrix effect and accuracy.

The method was completely described, fully validated and suitable for the studies of the determination of 4-chloroaniline in salmon fillet.

The Committee noted that some national authorities monitor diflubenzuron in fish fillet using LC-MS/MS methods that may be applicable for regulatory monitoring of diflubenzuron in salmon fillet. The LOQs are in the range 1–10 µg/kg.

4.1.2 Maximum residue limits

In recommending MRLs for diflubenzuron in salmon, the Committee considered the following factors:

- Diflubenzuron is authorized for use in salmon. The recommended dose is 3–6 mg/kg fish per day for 14 consecutive days, administered through feed. The withdrawal period is in the range 105–300 degree days.
- The ADI established by the Committee was 0–0.02 mg/kg bw.
- Diflubenzuron is the MR in tissues.
- The ratio of the concentration of MR to the concentration of total residue (TR) is 0.9 in salmon fillet.
- The MRL for salmon fillet based on the upper limit of the one-sided 95% confidence interval over the 95th percentile of residue concentrations (95/95 upper tolerance limit [UTL]) from the non-radiolabelled residue depletion study would allow estimation of an MRL of 590 µg/kg at the 117 degree days withdrawal period.
- Monitoring data from one Member State covering a period of 8 years (2010–2017) showed that 98.6% of the 641 samples analysed had no detectable residues. Detectable residues in Atlantic salmon were mainly in the range 1–2 µg/kg, with the highest concentration being 14 µg/kg.
- A validated analytical method for the determination of diflubenzuron in edible salmon tissues is available and may be used for monitoring purposes.
- The LOQ of the method for the determination of diflubenzuron is in the range 1–10 µg/kg.

In recommending MRLs for 4-chloroaniline in salmon, the Committee considered the following factors:

- 4-Chloroaniline is a metabolite of diflubenzuron in salmon.
- It may be present as an impurity in the diflubenzuron formulation.
- It may be produced from diflubenzuron during thermal food and feed processing.
- At the recommended withdrawal period of diflubenzuron (117 degree days), the concentrations of 4-chloroaniline in salmon fillet were below the LOD (0.33 µg/kg).
- A validated analytical method for the determination of 4-chloroaniline is available, with an LOQ of 1 µg/kg.
- Because 4-chloroaniline does not exhibit DNA-reactive genotoxicity in vivo, its estimated exposure can be compared with a TTC for Cramer Class III compounds (1.5 µg/kg bw per day).

Due to specific toxicological concerns, the risk assessment for diflubenzuron must consider both the parent compound and its contaminant and metabolite 4-chloroaniline, which may be present in a variety of foods and non-dietary sources.

Considering diflubenzuron itself, residue depletion data and the ADI of 0–0.02 mg/kg bw would permit recommending an MRL of 590 µg/kg. However, this MRL could result in estimates of exposure to 4-chloroaniline that may approach or even exceed the TTC for 4-chloroaniline if additional sources of exposure are considered.

While some occurrence data for 4-chloroaniline in diflubenzuron-treated fish are available, there is limited information on the origin of 4-chloroaniline in fish or its formation as a result of food processing. In addition, information about occurrence of 4-chloroaniline in other sources such as diflubenzuron-treated crops and consumer products is very limited. As a result, a comprehensive estimate of exposure cannot be derived.

The Committee concluded that even though an MRL of 590 µg/kg could be calculated for diflubenzuron in salmon, this needs to be reduced to ensure protection of consumers from effects due to exposure to 4-chloroaniline. The Committee therefore recommended an MRL for diflubenzuron in salmon of 10 µg/kg in muscle+skin in natural proportions, based on:

- data available to the Committee from monitoring of diflubenzuron in salmon, which showed that residues in salmon in the marketplace are typically very low (<2 µg/kg);

- analytical methods being available to determine residues of diflubenzuron as low as 1 μg/kg; and
- lower MRLs close to the LOQ being practicable in normal fish production.

Considering that the proposed MRL may not be consistent with all the approved withdrawal times, the Committee noted that some revision of existing product labelling of the formulations on the market may be required.

If it is possible to reduce the uncertainty around occurrence and exposure of 4-chloroaniline, it might be possible to increase the MRL for diflubenzuron in the future.

An addendum to the residue monograph was prepared.

4.2 Estimated dietary exposure

4.2.1 Diflubenzuron

Dietary exposure from pesticide residues

MRLs for diflubenzuron from its pesticide use have been set in a wide range of commodities including cereals (e.g. rice, wheat and barley), fruits (e.g. pome fruits, citrus fruits and stone fruits), animal products (e.g. meats, eggs, milks and offal), tree nuts, vegetables (e.g. mushrooms and peppers) and some fodder. MRLs range widely, from 0.01 mg/kg in rice to 20 mg/kg in dried chilli.

The international estimate of daily intake (IEDI) was calculated by JMPR for commodities of human consumption for which supervised trials median residues for diflubenzuron were available. The IEDI for the 13 Global Environment Monitoring System – Food Contamination Monitoring and Assessment Programme (GEMS/Food) cluster diets were 2–10% of the ADI. JMPR concluded that the exposure to diflubenzuron residues resulting from its proposed uses was unlikely to present a public health concern.

Dietary exposure from veterinary drug residues

At the 81st meeting, the Committee concluded that it was not necessary to establish an ARfD for diflubenzuron in view of its low acute oral toxicity and the absence of developmental toxicity or any other toxicological effects that would be likely to be elicited by a single dose *(1)*. This meeting reconfirmed this conclusion. Therefore, acute dietary exposure was not estimated.

Based on the new data made available, chronic dietary exposure was estimated based on the occurrence of diflubenzuron residues in salmon fillet. Dietary exposure estimates were based on incurred diflubenzuron median residues of 170 μg/kg in salmon fillet at 117 degree days withdrawal time

(according to currently approved good practice in the use of veterinary drugs) and an MR:TRR ratio of 0.9.

The global estimate of chronic dietary exposure (GECDE) for the general population was 0.84 µg/kg bw per day, which represented 4% of the upper bound of the ADI of 0.02 mg/kg bw per day. The GECDE for children was 2.85 µg/kg bw per day, 14% of the upper bound of the ADI.

Further estimates of chronic dietary exposure were carried out using national consumption data. Instead of using the highest mean and the highest 97.5th percentile consumption across surveys, the calculations were carried out using the mean and the highest reliable percentile for each individual national survey from available datasets (CIFOCOss) from which data on fish fillet consumption could be obtained.

The mean of 28 country-specific estimates for adults or the general population (genders combined) was 0.53 µg/kg bw per day (3% of the upper bound of the ADI), with a range of 0.02–0.84 µg/kg bw per day (<1–4% of the upper bound of the ADI).

The mean of 23 country-specific estimates for children was 1.15 µg/kg bw per day (6% of the upper bound of the ADI), with a range of 0.27–2.85 µg/kg bw per day (1–14% of the upper bound of the ADI).

The final recommended MRL of 10 µg/kg was not based on the UTL approach, and it is expected that with this MRL, the maximum dietary exposure to diflubenzuron will be at least an order of magnitude lower than the GECDE for all populations considered.

4.2.2 4-Chloroaniline

Dietary exposure to 4-chloroaniline from veterinary drug use

The level of risk associated with known uses of 4-chloroaniline was assessed by comparing estimated exposure with the Cramer Class III TTC value of 1.5 µg/kg bw per day.

When the source of 4-chloroaniline was assumed to be a veterinary drug, chronic dietary exposure was estimated based only on the potential occurrence of 4-chloroaniline in salmon fillet. Exposure was estimated based on the MRL of 10 µg/kg proposed for diflubenzuron, assuming that diflubenzuron is present in all fish fillet at its MRL, and that all of it is converted to 4-chloroaniline. This scenario is highly unlikely, and therefore these assumptions are highly protective of consumers.

The maximum amount of diflubenzuron theoretically present (i.e. 10 µg/kg) was adjusted for the difference in molecular weight between diflubenzuron (310.69 g/mol) and 4-chloroaniline (127.57 g/mol); therefore, the concentration used in the exposure estimate was 4 µg/kg. Fish fillet consumption was assumed

to be 268 g, which is the highest 97.5th percentile consumption for the general population (60 kg body weight) from available datasets (CIFOCOss).

In humans, possible adverse health effects of 4-chloroaniline, resulting from the metabolism of ingested diflubenzuron, would be covered by the ADI for diflubenzuron.

On this basis, dietary exposure to 4-chloroaniline from veterinary use of diflubenzuron was estimated to be 0.02 μg/kg bw per day for the general population, which represented 1% of the TTC for a Cramer Class III compound of 1.5 μg/kg bw per day.

Dietary exposure to 4-chloroaniline from pesticide use

No information was available on the occurrence of 4-chloroaniline in foods from pesticide use through its presence as an impurity, plant metabolism or food processing, and JMPR did not estimate dietary exposure to 4-chloroaniline. Consequently, dietary exposure could not be estimated by the Committee for commodities other than fish. However, the Committee acknowledges that use of diflubenzuron as a pesticide is a potential source of dietary exposure to 4-chloroaniline.

Exposure to 4-chloroaniline from consumer products

Exposure to 4-chloroaniline may occur from consumer products derived from the dye, textile, rubber and other industries *(23)*. Exposure to 4-chloroaniline from consumer products is not considered directly in a dietary exposure assessment of the compound; hence, in addition to exposure from the diet through veterinary drug and pesticide use, there will be potential exposure from non-dietary sources.

WHO carried out a concise international chemical assessment of 4-chloroaniline *(6)*. No reliable data on occupational exposure levels or exposure of the general population were available. They estimated exposure from dyed textiles (containing certain azo dyes), deodorant products (containing triclocarban) and mouthwashes (containing chlorhexidine). They also noted some additional routes of exposure in children, such as sucking of dyed textiles. It was concluded that total exposure by these routes was at most 0.3 μg/kg bw per day.

Overall exposure to 4-chloroaniline

The Committee noted that there are a variety of other potential sources of 4-chloroaniline exposure, including consumer products and dietary exposure from pesticide use. However, estimated exposure from fish is low (a maximum of 1.2% of the TTC) and is based on assumptions that highly overestimate actual exposure from salmon fillets. Therefore, the proposed MRL for diflubenzuron of 10 μg/kg in salmon muscle+skin in natural proportions is unlikely to pose a risk

from chronic exposure to 4-chloroaniline in humans. The Committee was unable to estimate overall exposure to 4-chloroaniline from all sources, but considered that the contribution from veterinary drug use would be small.

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Fosfomycin sodium

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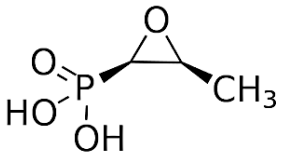
1. Explanation

Fosfomycin (International Union of Pure and Applied Chemistry [IUPAC] name: [(2R,3S)-3-methyloxiran-2-yl]phosphonic acid; Chemical Abstracts Service [CAS] registry number 23155-02-4) is a phosphoenolpyruvate analogue and an antibacterial substance produced by *Streptomyces fradiae*, *S. viridochromogenes* and *S. wedmorensis*; it can also be produced synthetically. It acts by inhibiting EC 2.5.1.7 (UDP-*N*-acetylglucosamine 1-carboxyvinyltransferase), thereby impairing bacterial cell wall synthesis. It has broad-spectrum antibacterial activity, and its use has not caused cross-resistance to other antibacterial substances. Fosfomycin has a phosphoric acid C-P bonded to an epoxypropyl group, but because it is unstable in the free state it exists as a sodium or calcium salt, depending on the pH. Fosfomycin calcium is used as an orally administered agent, and fosfomycin sodium is used as an injectable agent.

Fosfomycin is used as a veterinary medicine to treat *Escherichia coli*-related diarrhoea and salmonellosis in cattle, and pseudotuberculosis in

Perciformes. The disodium salt is used for intravenous, intramuscular and subcutaneous administrations, whereas the trometamol salt (tromethamine) and the calcium salt are used for oral administration.

Fosfomycin has not previously been evaluated by the Joint Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA). This evaluation was conducted at the request of the 24th session of the Codex Committee on Residues of Veterinary Drugs in Foods (CCRVDF) *(1)*.



Chemical structure of fosfomycin

A limited set of data was submitted by the sponsor to JECFA for the evaluation of fosfomycin. The Committee reviewed studies on the acute, repeated dose, reproductive and developmental toxicity, and the genotoxicity of fosfomycin sodium submitted by the sponsor. All toxicity tests on fosfomycin sodium submitted by the sponsor were performed via intraperitoneal or intravenous administration. In 2010, the Food Safety Commission (FSC) of Japan evaluated the acceptable daily intake (ADI) of fosfomycin calcium using data from oral administration tests *(2)*. Permission was obtained to review the original study reports for the purposes of this assessment *(3)*. In many of these studies, fosfomycin was administered by the oral route. To obtain additional information to support the evaluation, the Committee conducted a comprehensive review of the scientific literature (see Appendix 1 for details). The literature search resulted in 77 potentially relevant articles. Eight articles were considered relevant and were used in the monograph. None of the studies used for the evaluation indicated they were performed under good laboratory practice (GLP) conditions.

2. Biological data

2.1 Biochemical aspects

2.1.1 Absorption, distribution and excretion

(a) Kinetic study in animals

Rats (Donryu, male, 6–9 weeks old, two to four animals per group) were orally injected with unlabelled fosfomycin calcium or ^{3}H-labelled fosfomycin calcium

(solvent: 0.5% aqueous carboxymethylcellulose [CMC]sodium [CMC] solution) at a dose of 40 mg/kg body weight (bw) as fosfomycin. Blood, urine skin and tissue samples were collected; the concentrations of fosfomycin in each sample were quantified by bioassay and radioactivity measurement, and the absorption, distribution, metabolism and excretion were examined *(4)*.

The serum concentration of fosfomycin reached maximum concentration (C_{max}) (~13 μg/mL) 1–2 hours after administration. The urinary excretion (radiolabel values) was 50% at 4 hours after administration and 70% at 24 hours after administration. Based on these results, the estimated bioavailability of oral fosfomycin is at least 70%. Between 1 hour and 5 hours of oral administration, skin concentrations decreased remarkably. Fosfomycin was rapidly absorbed after administration, and was widely distributed to the body. Serum concentrations of fosfomycin decrease at the same time as tissue concentrations, and fosfomycin is rapidly excreted in urine.

In vitro absorption tests show that the absorption of fosfomycin in the intestine and colon is low, and absorption in the small intestine and caecum is high. Also, no differences were observed in the fosfomycin absorption rate in each portion of the small intestine (duodenum, jejunum and ileum). It may be concluded that orally administered fosfomycin is mainly absorbed in the small intestine. Also, the amounts of fosfomycin in the stomach, faeces and urine after 3 and 24 hours of the oral administration of fosfomycin calcium with ^{3}H marker measured with the scintigraphic count were remarkably similar to the value obtained with the bioassay. When thin-layer chromatography (TLC) was used to analyse urine samples 3 hours after administration, no metabolites were observed. It was concluded that fosfomycin is not metabolized in the body and excreted intact in urine.

An oral dose of fosfomycin calcium was administered in rats (Wistar male rats, five animals per group), rabbits (unknown breed, males and females, four to five animals per group) and dogs (mixed breed, females, eight animals per group) after about 17 hours fasting (20 and 40 mg/kg bw for rats, 20 mg/kg bw for rabbits and dogs) *(5)*. The study substance was administered in rats as a suspension (solvent: 0.5% water solution CMC) and as a water solution or suspension in rabbits and dogs. After administration, periodic blood, urine, skin and tissue samples were obtained, and the concentrations of each sample were measured using the bioassay method (agar plate method) to study absorption, distribution and excretion.

Twenty-four hours after dose administration, the excretion rate in urine in rats treated with 20 mg/kg bw was higher than in the group treated with 40 mg/kg bw; however, at a later time the excretion rate in the second group was higher than in the first group, and 72 hours after dose administration the cumulative values were 77.2% and 64.3%, respectively. Also, the excretion rate in

faeces was notably higher in the group treated with 40 mg/kg bw after 72 hours of administration, and the total excretion rate in both groups was 77.9% and 80.0%, respectively; no differences were found due to the higher or lower dose volume. From excretion in urine, the absorption level (comparatively) in the gastrointestinal tract is highest in rats, followed by dogs and then rabbits.

A single oral dose of fosfomycin was administered (as a dry syrup or in capsules with fosfomycin calcium, or as a powder of the active ingredient) to female dogs (beagles or mixed-breed dogs) with 17 hours fasting (the drug and the powder of the active ingredient have been tested) *(5)*. Periodic blood, urine, skin and tissue samples were obtained, and the concentrations in each sample were measured using a bioassay (agar plate method).

When an oral dose of fosfomycin calcium powder or syrup was administered (20 mg/kg bw), the average C_{max} in serum (measured value) was 19.4 and 18.4 µg/mL, respectively. The real C_{max} value was between 1 and 2 hours after dose administration. The average C_{max} in serum (measured value) of fosfomycin calcium administered as powder of the active ingredient or oral capsule (250 to 500 mg/capsule; 500 mg/animal) was 30.2 ug/mL for the active ingredient in powder: 29.5 µg/mL for 250 mg capsules, and 33.2 µg/mL for 500 mg capsules. The excretion in urine and faeces was tracked over time; activity in urine was recorded 24 and 48 hours after administration.

(b) Kinetic study in humans

The pharmacokinetics of fosfomycin disodium and tromethamine were examined after a single dose in 28 healthy adult subjects. Subjects received a single 1 hour intravenous infusion of 1 g and 8 g fosfomycin disodium, and a single dose of 3 g oral fosfomycin tromethamine in a phase I, randomized, open-label, three-period crossover study. After oral administration, C_{max} was 26.8 ±6.4 µg/mL, time to reach C_{max} (T_{max}) was 2.25 ±0.4 hours, area under the concentration–time curve from time 0 extrapolated to infinite time ($AUC_{0-\infty}$) was 191 ±57.6 µg/hour per mL, and the biological half-life ($t_{1/2}$) was 9.04 ±4.5 hours *(6)*.

The pharmacokinetics, safety and tolerability of two repeated dosing regimens of oral fosfomycin tromethamine were also evaluated in 18 healthy adult subjects. Subjects received 3 g every other day for three doses and then every day for seven doses, or vice versa, in a phase I, randomized, open-label, two-period crossover study. Serial blood (n = 11) and urine (n = 4 collection intervals) samples were collected before and up to 24 hours after dosing on days 1 and 5, along with pre-dose concentrations on days 3 and 7. Pharmacokinetic parameters were similar between days 1 and 5, both within and between dosing regimens *(7)*.

2.1.2 Bioavailability of each salt of fosfomycin

Fosfomycin calcium has relatively low oral bioavailability of 20% (12–37%) in humans compared with other salts such as sodium (41–85%) and tromethamine (33–44%), due to the inactivation by hydrolysis in the acidic gastric environment *(8-13)*.

Fosfomycin tromethamine is a more recent formulation that is highly hydrosoluble. The bioavailability of calcium and tromethamine salts of fosfomycin is reduced when taken orally following food. When fosfomycin tromethamine is taken under fasting conditions, serum concentrations of the tromethamine salt of fosfomycin are about twofold to fourfold higher than with the calcium formulation *(8, 14)*.

2.2 Toxicological studies

2.2.1 Acute toxicity

(a) Lethal doses

An acute toxicity test of fosfomycin sodium was conducted by the following administration routes – intravenous, intraperitoneal, intramuscular, subcutaneous and oral – using mice (4-week-old Institute of Cancer Research [ICR] strain mice, 10 males and 10 females each) and rats (5-week-old Wistar strain rats, 10 males and 10 females each). The median lethal dose (LD_{50}) values of fosfomycin sodium salt in mice are described in Table 1. Changes were similar in all groups of surviving animals; they included decreased respiratory rate and decreased locomotor activity. All animals that died upon being administered with fosfomycin sodium through the intravenous route died almost immediately (respiratory paralysis). Apart from adhesion and the effect on intra-abdominal organs caused by the intraperitoneal administration of fosfomycin sodium, histopathological examination of the surviving animals did not show any remarkable changes in mice or rats *(15)*.

In the acute toxicity tests using mice and rats, fosfomycin calcium was administered by intraperitoneal, subcutaneous and oral administration (Table 2). The LD_{50} for each administration in mice (ICR strain, 4-week-old, 10 males and 10 females) and rats (Wistar, 5-week-old, 10 males and 10 females) is shown in the table. During intraperitoneal administration, transient stretching position, respiratory rate decrease and locomotor decrease were observed in both male and female groups. The mice and rats suffered significant weight loss causing debilitation and death, which was most intense at 3–4 and 2–3 days after administration, respectively. At necropsy, adhesion of organs in the abdominal cavity and enlargement of the liver – considered to be due to local irritation of the drug by intraperitoneal administration – were observed. Subcutaneous

Table 1
LD_{50} for fosfomycin sodium in mice and rats (mg/kg body weight) n = 10

Animals (strain, age)	Administration	Male	Female	References
Mice (ICR, 4 weeks old)	Intravenous	1230	1225	*(15)*
	Intraperitoneal	2175	2467	
	Intramuscular	2625	2662	
	Subcutaneous	5100	6150	
	Oral	8020	7300	
Rats (Wistar, 5 weeks old)	Intravenous	1650	1560	
	Intraperitoneal	2060	2000	
	Intramuscular	2630	2460	
	Subcutaneous	5100	4320	
	Oral	4700	4550	

ICR: Institute of Cancer Research; LD50: median lethal dose.

Table 2
LD_{50} for fosfomycin calcium in mice and rats (mg/kg body weight) n = 10

Animals (strain, age)	Administration	Male	Female	References
Mice (ICR, 4 weeks old)	Intraperitoneal	994 (937.7~1053.6)	1029 (954.5~1109.3)	*(16)*
	Subcutaneous	>3500	>3500	
	Oral	>3500	>3500	
Rats (Wistar, 5 weeks old)	Intraperitoneal	1064	1036	
	Subcutaneous	>7000	>7000	
	Oral	>3500	>3500	

ICR: Institute of Cancer Research; LD50: median lethal dose.

administration showed no significant change in the general condition in both sets of animals. In the oral administration groups, transient mild locomotor depression, lacrimation, face-washing behaviour and vomiting-like behaviour were observed, but no deaths occurred in either the subcutaneous or the oral administration groups *(16)*.

2.2.2 Short-term studies of toxicity

(a) Mouse

Mice (ICR strain, 4 weeks old, 10 male and 10 female animals per group) were orally treated with fosfomycin calcium by gavage (0, 175, 350, 700, 1400 and 2800 mg/kg bw per day) for 35 days with a 1-day treatment break a week (equal to 0, 150, 300, 600, 1200 and 2400 mg/kg bw per day) *(16)*. There were nine in 20 lethal cases (males: 6/10; females: 3/10) and eight in 20 lethal cases (males: 4/10;

females: 4/10) in the groups treated at a rate of 1400 and 2800 mg/kg bw per day, respectively.

No clinical signs were observed, except for the presence of soft stools in animals of groups treated with doses higher than 1400 mg/kg bw per day from days 2 to 7 after starting administration until the end of the testing. Regarding body weight, in the group treated with 2800 mg/kg bw per day, from day 10 in males and day 21 in females onwards, inhibition of body weight gain in respect of the control group was observed. No changes were observed in the amount of food ingested after administration of fosfomycin calcium.

During necropsy of treated groups, distention of the ileum and caecum was observed. Regarding organ weight, a decrease was observed in absolute and relative weight of the spleen, as well as a decrease in absolute weight of the heart, kidney and testicle in the group treated at a rate of 2800 mg/kg bw per day, and a decrease in absolute and relative weight of the liver in the groups treated with doses higher than or equal to 350 mg/kg bw per day. In the case of females, a decrease was detected in the absolute and relative weight of the thymus in the group treated at a rate of 2800 mg/kg bw per day, as well as a decrease in absolute and relative weight of the liver in the groups treated with doses higher than or equal to 350 mg/kg bw per day. The histopathological analysis did not reveal any alterations related to ileum and caecal distension found during necropsy. One to three cases of vacuolization of hepatocytes in surviving males and females were observed in the groups treated with doses equal to or above 1400 mg/kg bw per day. Furthermore, two cases of infiltration of round cells in the liver were observed in females treated at a rate of 2800 mg/kg bw per day.

The no-observed-adverse-effect level (NOAEL) was estimated as 600 mg/kg bw per day, based on changes in vacuolization of hepatocytes at a dose of 1200 mg/kg of bw per day.

(b) **Rat**

Fosfomycin calcium was administered to rats (Wistar strain, 5 weeks old, 10 males and 10 females per group) at doses of 0, 175, 350, 700, 1400 and 2800 mg/kg bw per day) by gavage for 35 days (equal to 0, 150, 300, 600, 1200 and 2400 mg/kg bw per day) *(16)*.

Soft stool, diarrhoea and abdominal dilatation were observed in all dose groups. The dilatation was not considered a significant toxicological change because it was due to fluctuation of intestinal microflora in response to the administration of the antibacterial substance, and was rodent specific. The diarrhoea and soft stool might have been linked to dilatation, or could be related to a direct and inflammatory effect of fosfomycin on the gastrointestinal tract, mentioned below.

There were no treatment-related effects on body weight for feed consumption. In haematology, statistically significant findings were a decrease in red blood cells at 1400 and 2800 mg/kg bw, and decreases in haematocrit and haemoglobin at 2800 mg/kg bw in females, and a decrease in white blood cells at 2800 mg/kg bw in males. In blood biochemical parameters, statistically significant findings were increases in serum albumin and glucose at 1400 and 2800 mg/kg bw in males, an increase in aspartate aminotransferase (AST), a decrease in total cholesterol at 1400 and 2800 mg/kg bw, and increases in serum calcium and uric acid at 2800 mg/kg bw.

At necropsy, mild distention of the caecum was observed at autopsy in all treated groups. Changes in weight were observed in various organs: in males, increased absolute and relative weight of adrenals in males at 350 mg/kg bw and higher, increased relative liver weight in males at 700 mg/kg bw and higher, and decreased absolute and relative weights of spleen and heart at 1400 and 2800 mg/kg bw; in females, a decrease in absolute and relative weights of ovaries at 1400 and 2800 mg/kg bw, and decreases in relative weights of heart, spleen and kidney at 2800 mg/kg bw. Histopathological examination showed erosion, thickening and exfoliation of the glandular stomach mucosa in males and females in the group administered 175 mg/kg bw per day or more. Incidence (three in 20 animals, males and females combined) in vacuolated hepatocytes was increased in both sexes at 1400 and 2800 mg/kg bw. The observations in the gastrointestinal tract were not clearly dose dependent (range: 2–5 of 20 rats, males and females combined). The mild caecal distension observed during necropsies in groups treated in this testing is caused by alterations in intestinal flora due to antibiotic administration. Taking into consideration the particularities of the rodent caecum, this alteration was considered irrelevant for toxicological testing. The inflammatory reaction in the gastrointestinal tract was considered to be a direct and local effect, but the systemic toxicity after circulation in the blood was not. The Committee noted that there was inconsistency, in that the lesions induced by fosfomycin were not dose dependent, and there were no similar changes in the Wistar rats that underwent longer treatment by gavage up to 1400 mg/kg bw.

The Committee evaluated the NOAEL as 600 mg/kg bw, based on the increase in serum albumin, glucose and aspartate aminotransferase levels; decrease in total cholesterol level; decrease in absolute and relative weights of spleen and heart; and increase in the incidence of vacuolated hepatocytes at the dose of 1200 mg/kg bw.

Gavage oral administration of fosfomycin calcium was performed for 182 days using rats (Wistar strain, 5 weeks old, male, 10 animals per group) *(17)*. In this toxicity study, doses of 0, 87.5, 175, 350, 700 and 1400 mg/kg bw per day were administered, with 1 day/week withdrawal (equal to 0, 75, 150, 300, 600 and 1200 mg/kg bw per day). The number of deaths was one in 10 in the

87.5 and 350 mg/kg bw per day groups, two in 10 in the 700 mg/kg bw per day group, and three in 10 in the 1400 mg/kg bw per day group; all deaths were due to pneumonia. In the 350 mg/kg bw per day or less (several cases) group, soft faeces were observed 6 days after administration, and abdominal distension and diarrhoea were observed 13 days after administration; this continued for 1 month. In the 700 mg/kg bw per day or more administration group (5–6/20 cases), diarrhoea was observed 2–3 days after administration until the end of the test. In addition, in the 700 mg/kg bw per day or more administration groups, affected animals tended to scratch the whole body, but this was considered to be a transient reaction. There were no treatment-related effects on body weight and food consumption. Haematological and blood biochemistry tests showed decreased alkaline phosphatase and increased calcium and inorganic phosphate in the 1400 mg/kg bw per day group. No significant change was found in the urinary biochemistry test.

At necropsy, ileal and caecal distension was observed in six to eight cases in each group given 175 mg/kg bw or more per day. The mild caecal distension observed during necropsies in groups treated in this testing is caused by changes in intestinal flora due to antibiotic administration. Taking into consideration the particularities of the rodent caecum, this alteration was considered irrelevant for toxicological testing. In histopathology, mild vacuolation of hepatocytes was observed in three in 10 animals in the 1400 mg/kg bw per day group. In analysis with an ultramicroscope, hepatocytes showed mild reduction of mitochondria and accumulation of glycogen and vacuoles (glycogen efflux or smooth endoplasmic reticulum) in the 1400 mg/kg bw per day group.

The histopathological findings (vacuolation of hepatocytes) observed in the 1400 mg/kg bw per day group were considered to be effects resulting from fosfomycin administration. The NOAEL of this study was 600 mg/kg bw per day, based on vacuolated hepatocytes in males at 1200 mg/kg bw per day.

(c) **Rabbit**

A 35-day gavage oral administration of fosfomycin calcium was performed in rabbits (males, four animals per group) *(16)*. In this subacute toxicity study, doses of 0, 200 and 400 mg/kg bw per day were given, with 1 day off per week. In the haematological and blood biochemistry examinations, uric acid in each administration group and albumin in the 400 mg/kg bw per day group increased transiently 17 days after administration. After 35 days of administration, an increase in total cholesterol was observed in the 400 mg/kg bw per day group ($P < 0.05$). Biochemical examination of urine showed no significant change.

Necropsy and histopathological examination showed no obvious change due to fosfomycin calcium administration. Because an effect on cholesterol was

observed in the 400 mg/kg bw per day administration group, the male NOAEL in this study was estimated as 200 mg/kg bw per day. However, the number of animals was not adequate, and changes in clinical chemical data were not observed in an intravenous study at much higher doses. Therefore, this study was not considered for the risk assessment of fosfomycin residues in food.

(d) Dog

An oral toxicity test of fosfomycin calcium (0, 280 and 560 mg/kg bw per day) by gavage (with no treatment on Sundays, equal to 0, 240 and 480 mg/kg bw per day) was conducted in dogs (beagle, three females per group) *(16)*. There were no deaths during the study. In the 280 mg/kg bw per day group, excretion of watery faeces was observed in all cases from 2 days after administration until 14 days after administration, after which transition to soft faeces was observed. In the 560 mg/kg bw per day group, the same signs were observed until 17 days after administration, then soft faeces continued until the end of the test. A decrease in feed consumption and consequent decrease in body weight was found in one female in both the 260 and 560 mg/kg bw per day groups, but the decreases were transient. Haematological and blood biochemistry tests showed increases in calcium and inorganic phosphate in all dose groups. In addition, increases in aspartate aminotransferase (transient) and blood urea nitrogen were observed in the 560 mg/kg bw per day group. Biochemical examination of urine showed a temporary decrease in sodium in the 560 mg/kg bw per day group.

Scattered yellow nodules in the liver (1/3), mild thickening of the liver (1/3), renal congestion (2/3) and caecum distension were observed in the 280 mg/kg bw per day group. In the 560 mg/kg bw per day group, all cases showed mild thickening of the liver, renal congestion (1/3), renal atrophy (1/3) and caecal distension (1/3). Histopathological examination revealed slight swelling of tubular epithelial cells in the fosfomycin calcium administration group.

The NOAEL of this study could not be identified, and the lowest-observed-adverse-effect level (LOAEL) was estimated based on liver thickening, renal congestion, caecal distension and swelling of tubular epithelium in the male in the 240 mg/kg bw per day group.

2.2.3 Long-term studies of toxicity and carcinogenicity

The sponsor did not submit any data on long-term studies of toxicity and carcinogenicity.

2.2.4 Genotoxicity

(a) In vitro assay

In this test, six strains (TA1535–1538, TA98 and TA100) of *Salmonella typhimurium* were used. Tests were conducted with fosfomycin sodium solution at concentrations of 5.0×10^{-6} M/mL and 5.0×1.0^{-5} M/mL. Fosfomycin sodium showed no evidence of mutagenicity with any of the six tester strains, whereas the positive control (*N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, MNNG) showed induced reverse mutation for TA1535, TA100 and TA98. These results, summarized in Table 3, demonstrate that fosfomycin sodium has no mutability in bacteria *(18)*.

Testings using these six strains were also conducted using fosfomycin calcium at concentrations of 0.1, 0.3, 1, 3, 10 and 30 µg/plate. Fosfomycin calcium showed no evidence of mutagenicity with any of the six tester strains *(19, 20)*.

The yeast strain used was D5, which detects mainly mitotic recombination *(21)*. The rates of total aberrant colony formation were as follows: control (0.1 M phosphate buffer) –0.09%, fosfomycin sodium 5.0×10^{-6} M/mL –0.06%, 2.5×10^{-5} M/mL –0.17% and 1.25×10^{-4} M/mL –0.13%. There was no evidence for mitotic recombination with fosfomycin sodium, whereas the expected response was observed with the positive control mitomycin-C *(18)*. It was concluded that fosfomycin sodium was not genotoxic to *Saccharomyces cerevisiae* D5.

The mutability of fosfomycin sodium was studied using the *Bacillus subtilis* H17 Rec^+ and M45 Rec^- in vitro assay; JCL-ICR type specific-pathogen-free (SPF) mice aged 8–10 weeks were used for the in vivo assay. The in vitro assay showed no evidence of significant mutability with fosfomycin. The dominant lethal test showed a slight increase in the dominant lethal rate in the fosfomycin sodium group in weeks 1 and 5 compared with the control group, but the rate was zero at all other times, and the effects observed at weeks 1 and 5 were therefore considered incidental. The expected progressive increase in dominant lethal rate was observed with the positive control mitomycin-C. The results from the aforementioned Rec-assay tests and pre-pregnancy and early pregnancy administration (test 2) demonstrated that fosfomycin sodium has no mutability *(22)*.

(b) In vivo assay

The in vivo rodent dominant lethal test and the micronucleus test were negative, as shown in Table 4. It was considered that fosfomycin does not exhibit genotoxic effects *(24, 25)*.

Table 3
In vitro genotoxicity tests

Test	Strain	Dose	Result
Reverse mutation test *(19, 20)*	*Salmonella typhimurium* TA1535, TA1537, TA100, TA98, *Escherichia. coli* WP2 uvrA	0 (solvent control), 50, 150, 500, 1500, 5000 µg/mL (S9 ±) (fosfomycin sodium)	Negative
	Salmonella typhimurium Ta1535, Ta1537, Ta1538, Ta100, Ta98, *E. coli* Wp2 Uvra	0 (solvent control) 0.1, 0.3, 1, 3, 10, 30 µg/plate (S9 ±) (fosfomycin calcium)	Negative
DNA damage test (Rec-assay method)	*Bacillus subtilis* H17 Rec+M45 Rec-	5, 10, 100 µg/mL (fosfomycin sodium)	Negative
Mutation test (yeast) *(23)*			
Damage test (Rec-assay method) *(24)*	*Saccharomyces cerevisiae* D5 *(21)*	5×10^{-6}, 2.5×10^{-5}, 1.25×10^{-4} M/mL (fosfomycin sodium)	Negative

Table 4
In vivo genotoxicity tests

Test	Animals	Dose	Result
Dominant lethal test *(24)*	ICR male mice (8–10 weeks old)	0, 2000 mg/kg bw Single intraperitoneal administration; fosfomycin sodium	Negative
Micronucleus test *(26)*	ICR male mice (spleen and bone marrow cells)	0, 750, 1500 mg/kg bw Single intraperitoneal administration fosfomycin (salt unknown)	Negative

bw: body weight; ICR: Institute of Cancer Research.

2.2.5 Reproductive and developmental toxicity

(a) Organogenesis period administration test – rats

Oral doses of fosfomycin calcium were administered to pregnant rats (Wistar strain, 25–30 animals per group) by gavage between gestation days (GD) GD7 and GD17 (0, 140, 700 and 1400 mg/kg bw per day), and on GD20 embryos (F1) were removed by a caesarean section in two out of three of the cases for testing *(27)*. The remaining one out of three of the pregnant rats continued until natural birth to examine young development (F1), observing young behaviour (F1) during the fourth week of age.

No lethal cases in adult animals were observed. In general, although soft stools were observed in the groups treated with doses higher than or equal to 700 mg/kg bw per day, no inhibition of body weight gain or alterations in the amount of food ingested were detected. Temporary differences in the amount of water drunk were observed in treated groups compared with the control group.

No abnormalities in organs were observed on GD20 or the fourth week of age, or in fetal miscarriage rate.

Regarding effects on embryos and young (F1), although an increase was detected in the amount of resorptions in early fetal stage in the group treated at a rate of 1400 mg/kg bw per day ($P < 0.05$), no effects were observed on resorption frequency in later fetal stage, deaths in perinatal period, sex ratio in viable fetuses, body weight of fetuses, external appearance of fetuses or bone, or organic malformations attributable to dose administration. An increase in cases of delay in bone formation of sternebrae was observed in groups treated at a rate of 1400 mg/kg bw per day ($P < 0.05$). In the young, no effects were observed on number, main organs, body weight, weaning rate or behaviour.

Observations in this testing were dams' soft stools, an increment in the number of resorptions in early fetal stage, and a delay in bone formation in the group treated at a rate of 1400 mg/kg bw per day. Therefore, the NOAEL for both adult animals and fetuses was considered to be 700 mg/kg bw per day. Teratogenesis was not detected.

(b) Organogenesis period administration test – rabbits

Rabbits (Japanese white rabbit, 14 weeks old, seven animals per group) were orally administered fosfomycin calcium (0, 80, 140 and 420 mg/kg bw per day) at GD6–GD18 *(27)*. No cases of deaths or differences in body weight gain in dams were observed. Fetal miscarriages were observed in one in seven cases in each group. No effects were observed in fetal mortality, sex ratio in viable fetuses, fetal body weight, external appearance or bone formation of fetuses (F1) attributable to dose administration.

Based on these findings, the NOAEL for both maternal and embryo/fetus toxicity was set at 420 mg/kg bw per day, the highest dose tested. Fosfomycin was not teratogenic in rabbits (unpublished).

2.2.6 Special studies

(a) Central nervous system

Fosfomycin calcium was suspended in 1% gum arabic solution, and administered at doses of 40 mg/kg bw and 400 mg/kg bw. There was no effect on electroencephalogram (EEG) results in rabbits (three males), pentetrazol-induced convulsions in mice (10 males, ICR) and bemegride-induced convulsions in mice (10 males, ICR). Fosfomycin calcium was administered orally to mice (10 males per group, ICR), and no effects were observed in fighting behaviour, righting reflex, adaptability to inclined plate, anaesthetic prolongation action and antitremorine action *(28)*.

(b) **Peripheral nervous system**

Using rats (male), a phrenic nerve-diaphragm preparation was prepared, and nerves and muscles were stimulated to observe muscle contraction. Upon administration, the contraction response due to both stimuli was not changed by administration of 0.01% fosfomycin calcium, but was shorter at concentrations of 0.05–0.5%, although the contractions were mild *(28)*.

(c) **Cardiovascular and respiratory systems**

Fosfomycin had no effects on blood pressure or breathing of rabbits at doses of 1.0–80.0 mg/kg bw. No effect was also observed in an electrocardiogram (ECG) test of rabbit at 20–100 mg/kg, amplitude and pulse rate of guinea-pig at doses of 10^{-6}–10^{-3} g/mL (MAGNUS method), and amplitude and pulse rate of frog at doses of 10^{-7}–10^{-2} g/mL of fosfomycin *(28, 29)*.

(d) **Kidney function**

Fosfomycin at 50–200 mg/kg bw was administered orally to rats (Wistar strain) for 7 days. As a result, there was a transient increase in urinary sodium excretion compared with the control group, but almost no difference in body weight gain, urinary volume, urinary sodium, potassium excretion and urinary findings *(29)*.

(e) **Smooth muscle**

The effects of applying fosfomycin at 10^{-7}–10^{-3} g/mL in Tyrode solution on the motility of isolated guinea-pig and rabbit intestine were examined *(29)*. Fosfomycin did not affect the guinea-pig intestine. For the rabbit intestine, the amplitude of spontaneous contraction was increased by fosfomycin at 10^{-4} –10^{-3} g/mL, but no effect was observed on the intestinal contractile effects of acetylcholine, histamine dihydrochloride and barium chloride.

The effect of applying fosfomycin (no information given on the salt) at 10^{-7} to 2×10^{-3} g/mL in Ringer solution on the mobility of isolated guinea-pig trachea was observed. The application of fosfomycin at 2×10^{-3} g/mL resulted in a mild but reversible tonic reduction.

The effects of applying fosfomycin at 10^{-7} to 2×10^{-3} g/mL in Ringer-Locke solution on the automation of the isolated uterus of adult rats were observed. In the non-pregnant rat uterus, the application of fosfomycin at 10^{-3} to 2×10^{-3} g/mL resulted in reversible suppression of muscle tone and amplitude, but there were no effects on the automatic movement of the pregnant rat uterus.

(f) **Stomach**

Oral administration (100 and 400 mg/kg bw) of fosfomycin calcium (suspended in 1.0% gum arabic solution) to rats (Donryu, male, 10 animals per group) had

no effect on gastric fluid retention, gastric juice pH or free hydrochloric acid content. There was no significant effect on the gastric mucosa *(28)*.

(g) Immune responses

Fosfomycin calcium was administered with Freund's adjuvant to rabbits (male, three animals per group) subcutaneously in the back skin. No effect on antigen–antibody reaction was observed in precipitation reaction in agar, passive skin anaphylaxis (PCA) reaction and passive hemagglutination (PHA) tests *(28)*.

2.3 Microbiological effects

To determine the need to establish a microbiological ADI (mADI) for fosfomycin, the Committee used a decision-tree approach that was adopted by the 66th JECFA meeting *(30)*. This approach complies with the International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) guideline *(31-33)*.

The decision-tree approach first determines whether fosfomycin is microbiologically active and residues of fosfomycin and/or its metabolites are entering the human colon. If no microbiologically active fosfomycin residues are entering the colon, no mADI is necessary. If microbiologically active residues are present in the human colon, two end-points of public health concern must be considered: disruption of the colonization barrier, and increase in the population(s) of resistant bacteria. At Step 4 of the decision-tree process, it is possible to provide scientific justification to eliminate testing (i.e. the need for an mADI) for either one or both end-points. Step 5 is where an mADI is determined. Should an mADI not be necessary, the toxicological or pharmacological ADI is used.

The sponsor did not submit any data on the effects of fosfomycin on the intestinal microbiota. The Committee evaluated in vitro minimum inhibitory concentration (MIC) susceptibility testing data, in vivo human volunteer and laboratory animal tests, and antimicrobial resistance studies reported in the published scientific literature. The Committee completed a comprehensive literature search using several publicly accessible databases, as detailed in Appendix 1. In addition, the Committee evaluated the Food Safety Commission of Japan (FSCJ) April 2010 report on the risk assessment of fosfomycin (CAS No. 23155-02-4), using a set of data submitted to Japan's Ministry of Health, Labour and Welfare (MHLW) *(3)*. The data used for the risk assessment included pharmacokinetic tests of fosfomycin calcium in rats, rabbits, dogs, cows and yellowtails; residue tests of fosfomycin calcium in cows and yellowtails, and of fosfomycin sodium in cows; acute toxicity tests of fosfomycin calcium and

fosfomycin sodium in mice and rats; subacute toxicity tests in rats, rabbits and dogs; reproductive and developmental toxicity tests in rats and rabbits; genotoxicity tests; and microbiological effect tests.

The information and data derived from the literature search were used by the Committee to answer the following questions in the decision-tree for the assessment of fosfomycin.

Step 1: Are residues of the drug, and/or its metabolites, microbiologically active against representatives of the human intestinal microflora?

Yes, fosfomycin is a broad-spectrum antimicrobial that has bactericidal activity against both Gram-positive and Gram-negative bacteria *(34)*. In vitro susceptibility data suggest that fosfomycin activity against intestinal microbiota varied by species, from "not active" for *Bacteroides* spp. to "moderate activity" to "considerable activity" against other anaerobic intestinal bacteria. Facultative anaerobic bacteria such as *E. coli* and *Enterococcus* spp. are very susceptible to fosfomycin. In veterinary medicine, fosfomycin is used to treat infectious diseases in broiler chickens and pigs caused by *Salmonella* spp., *E. coli, Pasteurella* spp., *Staphylococcus* spp., *Streptococcus* spp., *Haemophilus* spp., *Klebsiella* spp. and *Pseudomonas* spp. Fosfomycin is used in human medicine worldwide for the treatment of uncomplicated urinary tract infections in women mainly due to susceptible strains of *E. coli* and *Enterococcus faecalis* and other uropathogens.

Fosfomycin is bactericidal and inhibits bacterial cell wall synthesis by inactivating the enzyme UDP-*N*-acetylglucosamine-3-enolpyruvyltransferase, also known as MurA *(34)*. This enzyme catalyses the committed step in peptidoglycan biosynthesis, namely the ligation of phosphoenolpyruvate to the 3'-hydroxyl group of UDP-*N*-acetylglucosamine. This pyruvate moiety provides the linker that bridges the glycan and peptide portion of peptidoglycan. Fosfomycin is a phosphoenol pyruvate analogue that inhibits MurA by alkylating the active enzymatic site cysteine residue (Cys 115) in *Escherichia coli*.

Fosfomycin is approved for use worldwide to treat uncomplicated urinary tract infections; thus, there are many published studies on the antimicrobial susceptibility to fosfomycin for the most common uropathogens (i.e. clinical isolates of *E. coli*, *Enterobacter* spp., *Staphylococcus* spp., *Proteus* spp., *Klebsiella* spp. and *Pseudomonas* spp.). However, the Committee focus is on the effects of ingested fosfomycin residues on commensal intestinal bacteria. Compared with the uropathogens, little is known of the susceptibility to fosfomycin of the commensal intestinal microbiota. Listed below are brief summaries of the published scientific literature on the in vitro testing for susceptibility of intestinal bacteria to fosfomycin.

Gutiérrez Altés and Noriega *(35)* determined the MICs for 98 strains of anaerobic bacteria against fosfomycin and other antimicrobials that came from clinical hospital specimens. Fosfomycin was inactive against *Bacteroides* spp. About 85–95% of the strains of *Peptococcus* and *Peptostreptococcus* were inhibited by 32 µg/mL of fosfomycin.

Forsgren and Walder *(36)* determined the MICs for 745 clinical isolates that included *Staphylococcus aureus* and *S. epidermidis,* group A and B *Streptococci*, *Streptococcus faecalis* and *Streptococcus pneumoniae, E. coli, Proteus mirabilis*, *Serratia marcescens*, *Haemophilus influenzae*, *Klebsiella*, *Enterobacter*, indole-positive *Proteus*, *Pseudomonas aeruginosa* and *Bacteroides fragilis.* All Gram-positive aerobic bacteria tested were sensitive and inhibited at fosfomycin levels of 64 µg/mL or less. Gram-negative aerobic bacteria were sensitive to fosfomycin and were inhibited at 64 µg/mL. The anaerobe *Bacteroides fragilis* was resistant to fosfomycin.

Jung *(37)* evaluated 50 strains of *Clostridium perfringens* isolated from human faecal specimens using a microtiter method for susceptibility to fosfomycin and other antibiotics. The MIC values for fosfomycin against *C. perfringens* ranged from 16 to 256 µg/mL with a geometric mean MIC of 48.3 µg/mL.

Lu et al. *(38)* studied the antimicrobial activity of fosfomycin against 960 strains of commonly encountered bacteria associated with urinary tract infections using standard agar dilution and disk diffusion methods. The bacteria studied included three common species of Enterobacteriaceae, Pseudomonas aeruginosa, Acinetobacter baumannii and Stenotrophomonas maltophilia; methicillin-susceptible and resistant S. aureus; and vancomycin-susceptible and resistant Enterococcus faecalis and E. faecium. Fosfomycin was highly active against E. coli, although a small number of strains appeared to have MICs that were similar to the calculated wild-type of 1 µg/mL. This MIC distribution, with a modal value of 0.5 µg/mL, differed markedly from that published by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) *(39)*, of 4 µg/mL.

Yamaguchi et al. *(40)* evaluated the fosfomycin susceptibilities of probiotic bacterial strains that are suggested to be effective for preventing antibiotics-associated diarrhoea. The MICs of fosfomycin and other antibiotics against probiotic strains were tested by the agar plate dilution method or broth microdilution method. Eight probiotic strains of Enterococcus faecalis, Bifidobacterium spp., Clostridium butyricum and Lactobacillus acidophilus were tested. Fosfomycin showed generally mild activity against Enterococci (MIC of 8–32 µg/mL) and anaerobic strains (*Bifidobacterium* spp. had an MIC of 32–64 µg/mL, *Clostridium butyricum* had an MIC of 32 µg/mL and *Lactobacillus acidophilus* had an MIC of >1024 µg/mL).

Rajenderan et al. *(41)* tested fosfomycin susceptibility of 211 *E. coli* (MIC_{50} of 0.5 µg/mL), 207 *Klebsiella* spp. (MIC_{50} of 8 µg/mL), 153 *Pseudomonas aeruginosa* (MIC_{50} of 32 µg/mL) and 354 *Acinetobacter* spp. from blood, urine and sputum samples by the broth microdilution method. Fosfomycin inhibited 90% of *E. coli* and *Klebsiella* spp. isolates.

Keepers et al. *(42)* compared fosfomycin susceptibility of 658 United States (US) urinary tract infection isolates with susceptibility to ciprofloxacin, levofloxacin, nitrofurantoin and trimethoprim/sulfamethoxazole (SXT). The isolates included E. coli (n = 257, MIC_{50} of 1 µg/mL), Klebsiella spp. (n = 156, MIC_{50} of 8 µg/mL), Enterobacter spp. (n = 79, MIC_{50} of 16 µg/mL), Pseudomonas aeruginosa (n = 60, MIC_{50} of 64 µg/mL), Enterococcus faecalis (n = 54, MIC_{50} of 32 µg/mL) and Proteus spp. (n = 52, MIC_{50} of 4 µg/mL). Of the 257 E. coli isolates, 99.6% were susceptible to fosfomycin.

Flamm et al. *(43)* reported on fosfomycin and comparators susceptibility, as tested against more than 2200 contemporary clinical isolates collected from US medical centres. Potent activity was exhibited against Gram-positive organisms, including *S. aureus* (MIC_{50} of 4 µg/mL). Activity against the intestinal bacteria anaerobes varied by species. Fosfomycin was active against Enterobacter, with an MIC_{50} of 4 µg/mL. All randomly selected *E. coli* isolated were susceptible to fosfomycin (MIC_{50} of 0.5 µg/mL).

The most potent activity was against *Veillonella* spp. (MIC values ranged from ≤0.03 to 0.06 µg/mL), *Finegoldia magna* (MIC_{50} of 0.5 µg/mL), *Clostridium* spp. (MIC_{50} of 32 µg/mL), *Peptostreptococcus* (MIC_{50} of 16 µg/mL), *Fusobacterium* (MIC_{50} of 8 µg/mL), *Prevotella* (MIC_{50} of >256 µg/mL), *Bacteroides fragilis* group (MIC_{50} of >256 µg/mL), *Propionibacterium* (MIC_{50} of >256 µg/mL); *Porphyromonas* spp. (MIC_{50} of >250 µg/mL), *Enterococcus faecalis* (MIC_{50} of 64 µg/mL) and *E. faecium* (MIC_{50} of 64 µg/mL).

The data used in the 2010 risk assessment from FSCJ were from a report of a comprehensive survey of the microbiological effects of antibacterial substances including fosfomycin (September 2006 to March 2007), against human clinical isolates before administration of antibiotics *(3)*. MICs were determined using standard Clinical and Laboratory Standard Institute methods *(44, 45)*. The report gave MIC values for facultative and strict anaerobic bacteria, as follows: *E. coli* (n = 30, MIC_{50} of 4 µg/mL), *Enterococcus* spp. (n = 30, MIC_{50} of 64 µg/mL), *Bacteroides* spp. (n = 30, MIC_{50} of >128 µg/mL), *Fusobacterium* spp. (n = 20, MIC_{50} of 8 µg/mL), *Bifidobacterium* spp. (n = 30, MIC_{50} of 64 µg/mL), *Eubacterium* spp. (n = 20, MIC_{50} of 64 µg/mL), *Clostridium* spp. (n = 30, MIC_{50} of 8 µg/mL), *Peptococcus* spp./*Peptostreptococcus* spp. (n = 30, MIC_{50} of 0.5 µg/mL), *Prevotella* spp. (n = 20, MIC_{50} of >128 µg/mL), *Lactobacillus* spp. (n = 30, MIC_{50} of >128 µg/mL) and *Propionibacterium* spp. (n = 30, MIC_{50} of >128 µg/mL) (Table 5).

Table 5

Summary of the MIC_{50} values for fosfomycin against bacterial strains representing human intestinal microbiota used in calculating the mADI

		MIC (µg/mL)		
		Fosfomycin		
Bacterial isolates	**No.**	**MIC_{50}**	**Range**	**Reference**
Facultative anaerobic bacteria	135	0.5	0.25–>256	Flamm et al. 2019 *(43)*
Escherichia coli	211	0.5		Rajenderan et al. 2014 *(41)*
	257	1		Keepers et al. 2017 *(42)*
	30	4	2–32	FSCJ
	207	64	16–>256	Flamm et al. 2019 *(43)*
Enterococcus spp.	30	64	8–128	FSCJ
	54	32		Keepers et al. 2017 *(42)*
Anaerobic bacteria	30	>128	>128	FSCJ
Bacteroides spp.	9	>256		Flamm et al. 2019 *(43)*
	20	8	4–16	FSCJ
Fusobacterium spp.	5	8		Flamm et al. 2019 *(43)*
	30	64	8–>128	FSCJ
Bifidobacterium spp.	20	64	16–>128	FSCJ
Eubacterium spp.	30	8	8–64	FSCJ
Clostridium spp.	5	32		Flamm et al. 2019 *(43)*
	30	0.5	0.5–>128	FSCJ
Peptococcus spp./*Peptostreptococcus* spp.	5	16		Flamm et al. 2019 *(43)*
Prevotella spp.	20	>128	>128	FSCJ
Lactobacillus spp.	30	>128	>128	FSCJ
Propionibacterium spp.	30	>128	>128	FSCJ

FSCJ: Food Safety Commission of Japan; mADI: microbiological ADI; MIC: minimum inhibitory concentration; spp.: species.

The mADI was calculated by FSCJ to be 0.019 mg/kg bw per day, based on the VICH guidelines *(31-33)*. This mADI was much lower than the toxicological ADI. Thus, FSCJ concluded that the ADI should be set at 0.019 mg/kg bw per day in the risk assessment of fosfomycin. The Committee adopted the 500 mL value, based on the volume measured in humans for use in the evaluation instead of 220 g *(33)*.

The in vivo human and animal studies are summarized below.

Hendlin et al. *(46)* determined the effect of oral fosfomycin calcium on several groups of microorganisms indigenous to the gut. Data from this double-blind study indicated that 2 g of fosfomycin given daily in four divided doses for 28 days increased the incidence of soft stools. Although there was no significant change in total coliforms, the numbers of *E. coli* decreased markedly from 10^5 to 10^2 organisms/g faeces. Concurrent with this decrease, the Klebsiella-

Enterobacter counts increased from 10^3 to 10^6 organisms/g faeces. Two weeks post-treatment, the *E. coli* and the Klebsiella-Enterobacter counts approached pretreatment levels. During the course of treatment, when the *E. coli* dropped precipitously, the few surviving *E. coli* still were susceptible to fosfomycin, as determined by an agar diffusion disc test.

Knothe et al. *(47)* determined the effect of daily dose at 10 g fosfomycin on the intestinal and pharyngeal flora in eight healthy male volunteers. The *E. coli* and *Enterobacter* spp. were markedly reduced during application and returned to normal in all subjects within 12 days or less. The total count of Enterococci was reduced by one to two decimal potencies under medication. There was no change in the counts of Bacteroides and Lactobacteria. No selection of *C. difficile* was observed.

Naber et al. *(48)* reviewed the intestinal microbiome of mice that received either a single dose of fosfomycin, 7 days of nitrofurantoin, water as a substance-free vehicle, or two different doses of the phytocombination. Although the therapy with fosfomycin or nitrofurantoin showed massive shifts in the microbiome, the intestinal bacteria remained largely unaffected by the phytocombination. Alpha-diversity – a measure of the biodiversity of bacteria – was found in the faeces of the phytotherapeutically treated mice on a level with the faeces of the mice that had only received water. This was even the case when the plant combination was administered in a 10-fold higher than human equivalent dosage. The faeces of the nitrofurantoin-treated mice, however, were clearly outside the normal range. This difference was even more pronounced in the mice who received single dose therapy with fosfomycin, and some bacterial families completely disappeared.

Fosfomycin is widely used in pig farms for the treatment of a wide variety of bacterial infections. A study by Fernández Paggi et al. *(49)* analysed the elimination of fosfomycin disodium in colostrum or milk of the sow, and the impact of ingested fosfomycin concentrations in colostrum on the microbiota and intestinal morphophysiology of suckling piglets. Intestinal contents from the jejunum-ileum, caecum and colon of the suckling pig were monitored for Enterobacteriaceae and Lactobacillus on selective media, and enumerated by plate counts. There were no statistically significant differences in the Enterobacter/Lactobacillus ratio between control piglets and fosfomycin-treated piglets. An absence of diarrhoea also suggested that the intestinal microbiota was not disrupted. Overall, the studied concentrations did not produce imbalances in the microbiota or morphophysiology of the gastrointestinal tract of the piglet; this finding correlates with the in vitro susceptibility data for fosfomycin against human intestinal microbiota.

Step 2: Do residues enter the human colon?

Yes, fosfomycin residues could enter the colon of a person ingesting tissues

from treated food-producing animals. Pharmacokinetic studies in experimental animal models and in humans indicated that, after oral administration, fosfomycin is apparently not metabolized and excreted unchanged in the urine and faeces in active form. The bioavailability of fosfomycin varies with the type of formulation, route of administration and animal species; fosfomycin calcium has relatively low oral bioavailability of 20% (12–37%) in humans compared with other salts such as sodium (41–85%) and tromethamine (40%, 33–44%), due to inactivation by hydrolysis in the acidic gastric environment *(8-13)*. The bioavailability of calcium and tromethamine salts of fosfomycin is reduced when taken orally following food. In addition, when taken under fasting conditions, serum concentrations of fosfomycin with the tromethamine salt are about twofold to fourfold higher than those with the calcium formulation *(8, 14)*. Based on data on the urinary excretion of fosfomycin calcium by humans (Section 2.1.2), the Committee determined that the bioavailability is about 20%. Therefore, the value to be used in the formula for the fraction of the oral dose available to microorganisms is estimated to be 0.80 (i.e. 1 – 0.20).

Step 3: Do the residues entering the human colon remain microbiologically active?

Yes. Many animal species and humans do not metabolize fosfomycin. Hence, fosfomycin residues would remain microbiologically active in the gastrointestinal tract.

Step 4: Is there any scientific justification to eliminate testing for either one or both end-points of concern (i.e. disruption of the colonization barrier or resistance development)?

No. Multiple mechanisms of fosfomycin resistance have been reported *(34, 50)*. One mechanism is based on decreased uptake by the bacterium due to mutations in the genes that encode the glycerol-3-phosphate transporter or the glucose-6-phosphate transporter. Another mechanism is based on point mutations in the binding site of the targeted enzyme (MurA), and several isolates of *E. coli* have clinical resistance levels (32 μg/mL) due to increased expression of the MurA gene. A third mechanism of resistance is based on the inactivation of fosfomycin, either by enzymatic cleavage of the epoxide ring or by phosphorylation of the phosphonate group. Fosfomycin dosing regimens that include a total daily dose of up to 24 g per day resulted in the emergence of a resistant subpopulation within 30–40 hours of drug exposure, suggesting that resistance can occur rapidly.

For the antimicrobial resistance development end-point, despite the use of fosfomycin in human medicine, the prevalence of fosfomycin resistance in clinical isolates of Enterobacteriaceae from urinary tract infections remains

relatively low *(51, 52)*. However, no data were provided by the sponsor on antimicrobial resistance related to exposure to residue levels of fosfomycin in the gastrointestinal tract. The Committee's review of the published scientific literature did not reveal any reports that fosfomycin at residue levels can select for the emergence of resistance in intestinal microbiota. Therefore, it was not possible to determine the resistance development end-point of concern. This is a data gap that should be evaluated. Although the resistance frequency rate is relatively low in *E. coli* strains (1–3%) and other Enterobacteriaceae species, studies indicate that resistance is mainly mediated by plasmid encoded *fos* genes; hence, there is potential for plasmids in these bacteria to be transferred to other intestinal microbiota and act as a reservoir of fosfomycin-resistant bacteria in the gastrointestinal tract. For the in vivo studies to determine colonization barrier disruption, the concentration levels of fosfomycin typically used in the animal and human volunteer studies were single-dose treatments at therapeutic concentration levels; thus, it was not possible to determine a no-effect dose. However, in vitro fosfomycin susceptibility data indicated a range of activities from negligible to moderate to high, depending on the intestinal bacteria genus and species tested (Table 5). Therefore, the end-point of concern for fosfomycin is the disruption of the colonization barrier using MIC data. Consequently, there is a need to determine an mADI for fosfomycin residues.

Step 5: Derivation of an mADI using the VICH GL36 guideline approach

The formula for deriving the upper bound of the mADI for the end-point of disruption of the colonization barrier is as follows:

$$\text{mADI} = \frac{\text{MIC}_{\text{calc}} \times \text{Mass of colon content}}{\text{Fraction of oral dose available to microorganisms} \times \text{Body weight}}$$

where:

- MIC_{calc} is derived from the lower 90% confidence limit for the mean MIC_{50} of the relevant genera for which the drug is active (as described in Appendix C of VICH GL36 (33)). The MIC_{calc} is derived as follows:

$$\frac{Std\ dev}{Lower\ 90\%\ CL} = \text{Mean MIC}_{50} - \sqrt{n} \times t0.10, \text{df}$$

where:

- Mean MIC_{50} is the mean of the log-transformed MIC_{50} values;
- Std dev is the standard deviation of the log-transformed MIC_{50} values;
- n is the number of MIC_{50} values used in the calculations; and

- $t_{0.10,df}$ is the 90th percentile from a central t-distribution with degrees of freedom (df) = $n-1$.

Mass of colon volume: The Committee adopted the 500 mL value, based on the volume measured in humans for use in the evaluation instead of 220 g *(33)*.

Fraction of oral dose available to microorganisms: It is recommended that this value be based on in vivo measurements for the drug administered orally. Alternatively, if sufficient data are available, this value can be calculated as 1 minus the fraction of an oral dose excreted in urine. The fraction may be lowered if quantitative data (in vitro or in vivo) show that the drug is inactivated during transit through the intestine. Human data are best, but if no such data are available, non-ruminant animal data are recommended. In the absence of data to the contrary, it should be assumed that metabolites have antimicrobial activity equal to the parent compound.

Based on the bioavailability of about 20% in humans, the fraction of oral dose of fosfomycin available in the colon is 1 – 0.20 = 0.80.

Body weight: An adult human is assumed to weigh 60 kg.

Therefore, the upper bound of the mADI for fosfomycin is calculated as follows:

$$\text{Upper bound of mADI} = \frac{0.00258 \text{ mg/mL} \times 500 \text{ mL}}{0.80 \times 60 \text{ kg bw}} = \textbf{0.0269 mg/kg bw per day}$$

The MIC_{calc} of 2.58 µg/mL is based on the MIC_{50} for *E. coli* (0.5 µg/mL), *Enterococcus* (32 µg/mL), *Fusobacterium* (8 µg/mL), *Bifidobacterium* (64 µg/mL), *Eubacterium* (64 µg/mL), *Clostridium* (8 µg/mL), and *Peptococcus*/*Peptostreptococcus* (0.5 µg/mL) (Table 5).

For the effects on the colonization barrier, an mADI of 0–0.0269 mg/kg bw was established.

The microbiological acute reference dose (mARfD) for fosfomycin was determined using the following formula:

$$\text{Upper bound of mADI} = \frac{0.00258 \text{ mg/mL} \times 500 \text{ mL}}{0.80 \times 60 \text{ kg bw}} = \textbf{0.0269 mg/kg bw per day}$$

$$= \frac{0.00258 \text{ mg/mL} \times 3 \times 500 \text{ mL}}{0.80 \times 60 \text{ kg bw}} = \textbf{0.0807 mg/kg bw per day}$$

For the effects on the colonization barrier, an mARfD of 0.0807 mg/kg bw was established.

2.4 Observations in humans

The sponsor did not submit any relevant data on fosfomycin and humans.

Fosfomycin continues to be used clinically (normally 2–4 g per human per day, equivalent to 40–80 mg/kg bw per day of fosfomycin sodium), with the major route being intravenous. After treatment with fosfomycin by oral administration, mild and self-limited gastrointestinal disturbances, such as diarrhoea (including from *C. difficile*), nausea, abdominal pain and dyspepsia are reported as the most common adverse events. Headaches, dizziness, infections of the upper respiratory tract, vaginitis, and bacterial or fungal superinfections have also been reported. In a French study, hypokalaemia was reported in 26% of patients (19/72) *(34)*. Although potassium was administered in all patients, hypokalaemia was found only when fosfomycin was administered in 30–60-minute infusions, but did not occur when the period of administration was extended to 4 hours. Other adverse events reported in that study were infusion site reactions (4%), heart failure and hypertension due to sodium overload (6%), and a 1% increase in alanine aminotransferase *(34, 53)*.

Adverse events associated with fosfomycin use were also reviewed from 23 trials (eight comparative and 15 non-comparative) of parenteral administration of fosfomycin in 1242 patients. For oral fosfomycin, prospective comparative trials (n = 28) in 2743 patients were included. The most frequent adverse effects associated with parenteral fosfomycin were rash, peripheral phlebitis, hypokalaemia and gastrointestinal disorders. The incidence of side-effects was found to start at a daily dose of more than 2 g, and was not dependent on the duration of administration *(54)*.

In three US trials, 1233 patients were treated with Monurol (fosfomycin tromethamine). The most frequently reported adverse events – which occurred in more than 1% of the study population, regardless of drug relationship – were diarrhoea (10.4%), headache (10.3%), vaginitis (7.6%), nausea (5.2%), rhinitis (4.5%), back pain (3.0%), dysmenorrhoea (2.6%), pharyngitis (2.5%), dizziness (2.3%), abdominal pain (2.2%), pain (2.2%), dyspepsia (1.8%), asthenia (1.7%) and rash (1.4%). In addition, adverse events occurring in clinical trials at a rate of less than 1%, regardless of drug relationship, were abnormal stools, anorexia, constipation, dry mouth, dysuria, ear disorder, fever, flatulence, flu syndrome, haematuria, infection, insomnia, lymphadenopathy, menstrual disorder, migraine, myalgia, nervousness, paraesthesia, pruritus, skin disorder and vomiting. In the US study population, statistically significant laboratory changes reported without regard to causal relationship included increased eosinophil count, increased or decreased white blood cell count, increased bilirubin, increased alanine aminotransferase, increased aspartate aminotransferase, increased alkaline phosphatase, decreased haematocrit, decreased haemoglobin,

and increased or decreased platelet count. The changes were generally transient, were not clinically significant and occurred in less than 1% of patients. In the same study population, adverse events that were considered to be drug related by the investigators and reported in greater than 1% of the fosfomycin-treated patients were diarrhoea (9.0%), vaginitis (5.5%), nausea (4.1%), headache (3.9%), dizziness (1.3%), asthenia (1.1%) and dyspepsia (1.1%). The most frequently observed symptom, diarrhoea, was considered mild and self-limiting *(55)*.

3. Comments

3.1 Biochemical data

Rats received unlabelled or ^{3}H-labelled fosfomycin calcium by oral gavage at a dose of 40 mg/kg bw. The serum concentration of fosfomycin reached C_{max} (~13 µg/mL) 1–2 hours after administration. Urinary excretion was 50% by 4 hours and 70% by 24 hours after administration. Fosfomycin was rapidly absorbed after oral administration and was widely distributed in the body.

In vitro studies showed that the absorption of fosfomycin in the large intestine (except in the cecum) is low, but in the small intestine it is high.

Following an oral dose of fosfomycin calcium to rats, rabbits and dogs at 20 mg/kg bw *(5)*, based on urinary excretion data, the extent of absorption was in the rank order of rats, greater than dogs, greater than rabbits.

Following oral administration of fosfomycin calcium to dogs, C_{max} in serum increased less than proportionately with dose, being 19 µg/mL at 20 mg/kg bw (time to reach the maximum concentration [T_{max}] 1–2 hours), 29.5 µg/mL at 250 mg and 33.2 µg/mL at 500 mg *(56)*.

The pharmacokinetics of fosfomycin tromethamine were determined following a single oral dose of 3 g to healthy adult subjects. C_{max} was 26.8 ±6.4 µg/mL, T_{max} was 2.25 ±0.4 hours, area under the concentration–time curve from time 0 extrapolated to infinite time ($AUC_{0-\infty}$) was 191 ±57.6 µg/hour per mL, and $t_{1/2}$ was 9.04 ±4.5 hours *(6)*. The bioavailability of fosfomycin is low, in part due to hydrolysis in the acidic gastric environment. In humans and animals, fosfomycin calcium has an oral bioavailability of about 20% (12–37%), which is lower than that of other salts such as disodium (41–85%) and tromethamine (~40%, 33–44%) *(8-11, 13, 57)*. The oral bioavailability of the calcium and tromethamine salts of fosfomycin is reduced when taken following food. There is no evidence that fosfomycin undergoes any metabolism in humans or laboratory species.

3.2 Toxicological data

All toxicity tests with fosfomycin disodium submitted by the sponsor were performed following intraperitoneal or intravenous administration. Therefore, information on other salts, particularly the calcium salt administered by the oral route, was used to help complete the toxicological evaluation.

In rats and mice, the oral median lethal dose (LD_{50}) of fosfomycin disodium was more than 4500 mg/kg bw. The LD_{50} after intravenous administration was lower, but was still more than 1200 mg/kg bw. The LD_{50}s following other routes of administration (intramuscular and intraperitoneal) were of intermediate value *(58)*.

Repeat dose studies of toxicity were conducted in mice (35 days), rats (35 days and 182 days) and dogs (182 days). On repeated administration by the oral route, effects related to direct antimicrobial activity in the gastrointestinal tract were often observed. These were not considered a suitable basis for the assessment of systemic toxicity; such effects are more appropriately covered under the microbiological evaluation. On repeat administration, consistent systemic effects were seen on the liver; in the dog, the kidney was also a target.

In a 35-day toxicity study, mice were administered fosfomycin calcium by oral gavage at doses (as fosfomycin acid) of 0, 175, 350, 700, 1400 and 2800 mg/kg bw per day for 6 days per week (equal to 0, 150, 300, 600, 1200 and 2400 mg/kg bw per day) *(58)*. The NOAEL was 600 mg/kg bw per day, based on vacuolization of hepatocytes at 1200 mg/kg bw per day.

In a 35-day toxicity study, rats were administered fosfomycin calcium by oral gavage at doses (as fosfomycin acid) of 0, 175, 350, 700, 1400 and 2800 mg/kg bw per day for 6 days per week (equal to 0, 150, 300, 600, 1200 and 2400 mg/kg bw per day) *(58)*. The NOAEL was 600 mg/kg bw per day, based on increases in serum albumin, glucose, aspartate transaminase and total cholesterol, and an increased incidence of vacuolated hepatocytes at 1200 mg/kg bw per day.

In a 182-day toxicity study, rats were administered fosfomycin calcium by oral gavage at doses (as fosfomycin acid) of 0, 87.5, 175, 350, 700 and 1400 mg/kg bw per day for 6 days per week (equal to 0, 75, 150, 300, 600 and 1200 mg/kg bw per day) *(59)*. The NOAEL was 600 mg/kg bw per day, based on vacuolation of hepatocytes at 1200 mg/kg bw per day.

In a 182-day toxicity study, dogs were administered fosfomycin calcium by oral gavage at doses (as fosfomycin acid) of 0, 280 and 560 mg/kg bw per day for 6 days per week (equal to 0, 240 and 480 mg/kg bw per day) *(58)*. No NOAEL could be identified for this study, because decreased feed consumption and body weight, increases in plasma calcium and phosphate, mild liver thickening and renal congestion were observed at 240 mg/kg bw per day, the lowest dose tested.

It is possible that these effects were secondary to antimicrobial activity in the gastrointestinal tract, but no specific information on this was available.

No long-term studies of toxicity and/or carcinogenicity were submitted by the sponsor, and no such information could be identified in a search of the open literature.

The genotoxic potential of fosfomycin, largely as the disodium salt, was investigated in an adequate range of in vitro and in vivo assays. No evidence of genotoxicity was observed.

The Committee concluded that fosfomycin is unlikely to be genotoxic.

As fosfomycin is unlikely to be genotoxic, any carcinogenicity would be secondary to prolonged preneoplastic damage – for which there was no indication in a repeat dose (182-day) study in rats – and because the toxicity that was observed did not progress in severity from 35 to 182 days, the Committee concluded that fosfomycin is unlikely to pose a carcinogenic risk to humans.

In a two-generation study of reproductive toxicity, rats were administered fosfomycin disodium intraperitoneally at doses of 250, 750 and 1500 mg/kg bw per day *(60)*. The NOAEL for parental toxicity was 750 mg/kg bw per day, based on a reduction in spontaneous locomotor activity, loose stools, hepatic thickening and changes in other abdominal organs at 1500 mg/kg bw per day. The Committee noted that these effects might have been secondary to the route of administration. The NOAEL for reproductive toxicity was 1500 mg/kg bw per day, the highest dose tested. The NOAEL for offspring toxicity was 1500 mg/kg bw per day, the highest dose tested.

In a study of developmental toxicity, rats were administered fosfomycin calcium by oral gavage from gestational day (GD)7 to GD17 at doses (as fosfomycin acid) of 0, 140, 700 and 1400 mg/kg bw per day *(61)*. The NOAEL for maternal toxicity was 700 mg/kg bw per day, based on an increase in early resorptions at 1400 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was also 700 mg/kg bw per day, based on a delay in sternebrae formation at 1400 mg/kg bw per day.

In a study of developmental toxicity, rabbits were administered fosfomycin calcium by oral gavage from GD6 to GD18 at doses (as fosfomycin acid) of 0, 80, 140 and 420 mg/kg bw per day *(61)*. The NOAELs for maternal and for embryo/fetal toxicity were both 420 mg/kg bw per day, the highest dose tested.

In a study of developmental toxicity, rabbits were administered fosfomycin disodium intravenously from GD6 to GD18 at doses (as fosfomycin acid) of 0, 80, 100, 200, 400 and 800 mg/kg bw per day *(62)*. The NOAEL for maternal toxicity was 800 mg/kg bw per day, the highest dose tested. The NOAEL for embryo/fetal toxicity was 400 mg/kg bw per day, based on a reduction in the body weight of fetuses at 800 mg/kg bw per day.

The Committee concluded that fosfomycin is not teratogenic.

Observations in humans

In human subjects receiving fosfomycin in clinical trials, the most common (9%) side-effect reported was diarrhoea. Other side-effects reported with a frequency of less than or equal to 5% were relatively nonspecific.

3.3 Microbiological data

A decision-tree approach adopted by the 66th meeting of the Committee (30) that complies with VICH GL36 (31-33) was used by the Committee to determine the need for and to establish, if necessary, an mADI for fosfomycin. In addition, the Committee determined the need for a microbiological ARfD (mARfD) *(63)*.

The sponsor did not submit any data on the effects of fosfomycin on the intestinal microbiota. The Committee evaluated data from in vitro MIC susceptibility studies, in vivo human volunteer and laboratory animal studies, and antimicrobial resistance studies reported in the published scientific literature. The Committee used the information and data derived from the literature search to answer the following questions in the decision tree for the assessment of fosfomycin.

Step 1: Are residues of the drug, and/or its metabolites, microbiologically active against representatives of the human intestinal microflora?

Yes, fosfomycin is a broad-spectrum antimicrobial that has bactericidal activity against both Gram-positive and Gram-negative bacteria *(34)*. In vitro susceptibility data suggest that fosfomycin activity against intestinal microbiota varies by species, from no activity against *Bacteriodes* spp, to moderate to considerable activity against other anaerobic intestinal bacteria. Facultative anaerobic bacteria such as *E. coli* and *Enterococcus* spp. are very susceptible to fosfomycin.

Step 2: Do residues enter the human colon?

Yes, fosfomycin residues could enter the colon of a person ingesting tissues from treated food-producing animals. Fosfomycin is not metabolized and is excreted unchanged in the urine and faeces in the active form.

Step 3: Do the residues entering the human colon remain microbiologically active?

Yes. Humans do not metabolize fosfomycin. Hence, fosfomycin residues would remain microbiologically active in the gastrointestinal tract.

Step 4: Is there any scientific justification to eliminate testing for either one or both end-points of concern – i.e. disruption of the colonization barrier or resistance development?

No. For the colonization barrier disruption end-point, in vitro fosfomycin susceptibility data indicated activity against a number of intestinal bacteria genera and species. For the antimicrobial resistance development end-point, despite its use in human medicine, the prevalence of fosfomycin resistance in clinical isolates of *Enterobacteriaceae* from urinary tract infections remains relatively low *(51, 52)*. However, no data were provided by the sponsor on the effects of exposure to residue levels of fosfomycin in the gastrointestinal tract on antimicrobial resistance. A review of the published scientific literature by the Committee did not reveal any reports on studies of selection for the emergence of resistance in intestinal microbiota by residue levels of fosfomycin. Therefore, the resistance development end-point of concern could not be assessed by the Committee. Although the resistance frequency rate is relatively low in *E. coli* strains (1–3%) and other *Enterobacteriaceae* species, resistance in such species is mediated mainly by plasmid encoded *fos* genes *(34, 64)*. There is potential for the plasmids in these bacteria to be transferred to other intestinal microbiota, and hence for them to serve as a reservoir of fosfomycin-resistant bacteria in the gastrointestinal tract. The absence of information on the selection for and emergence of resistance in the microbiota in the gastrointestinal tract is an important data gap that needs to be assessed before the evaluation of fosfomycin can be completed.

Step 5: Derivation of an mADI using the VICH GL36 guideline approach

The formula for deriving the upper bound of the mADI for disruption of the colonization barrier is as follows:

$$\text{mADI} = \frac{\text{MIC}_{calc} \times \text{Mass of colon content}}{\text{Fraction of oral dose available to microorganisms} \times \text{Body weight}}$$

where:

- MIC_{calc}: In accordance with Appendix C of VICH GL36 (31-33), calculation of the estimated NOAEC (MIC_{calc}) for colonization barrier disruption uses MIC values from the lower 90% confidence limit of the mean MIC_{50} for the most relevant and sensitive human colonic bacterial genera. The MIC_{calc} was 0.00258 mg/mL.
- *Mass of colon content*: The 500 mL value is based on the colon volume measured in humans (33).

- *Fraction of oral dose available to the microorganisms*: Fosfomycin is rapidly absorbed and is excreted unchanged in urine and faeces. Based on data on the urinary excretion of fosfomycin calcium in humans, the oral bioavailability is about 20%. Therefore, 1 – 0.20 = 0.80 provides an estimate of the value to be used in the formula for the fraction of the oral dose available to intestinal microorganisms.
- The *body weight* of an adult human is assumed to be 60 kg.

The upper bound of the mADI for disruption of the colonization barrier by fosfomycin was calculated as follows:

$$\text{Upper bound of mADI} = \frac{0.00258 \text{ mg/mL} \times 500 \text{ mL}}{0.80 \times 60 \text{ kg bw}} = \textbf{0.0269 mg/kg bw per day}$$

In the absence of information on microbial resistance, it was not possible to determine an overall mADI for fosfomycin.

The microbiological end-point of concern for acute exposure is colonization barrier disruption. The mARfD for fosfomycin was therefore determined using the following formula:

$$\text{Upper bound of mADI} = \frac{0.00258 \text{ mg/mL} \times 500 \text{ mL}}{0.80 \times 60 \text{ kg bw}} = \textbf{0.0269 mg/kg bw per day}$$

$$= \frac{0.00258 \text{ mg/mL} \times 3 \times 500 \text{ mL}}{0.80 \times 60 \text{ kg bw}} = \textbf{0.0807 mg/kg bw per day}$$

- A *correction factor* of 3 was used to allow for temporal dilution during gastrointestinal transit and for dilution by consumption of additional meals.
- Other terms are as described above for the upper bound of the mADI for disruption of the colonization barrier.

4. Evaluation

The Committee determined a toxicological ADI for fosfomycin (as the acid) of 0–0.48 mg/kg bw on the basis of a LOAEL of 240 mg/kg bw per day, for decreased feed consumption and body weight, increases in plasma calcium and inorganic phosphate, mild liver thickening and renal congestion in a 182-day repeat dose toxicity study in dogs, with application of a safety factor of 500 to account

Table 6
Studies relevant to risk assessment

Species/study type	Route of administration	Fosfomycin salt	Doses	Critical end-point	NOAEL (mg/kg bw per day)	LOAEL (mg/kg bw per day)
Mouse						
35 days subacute toxicity test	Oral	Calcium	0, 175, 350, 700, 1400 and 2800 mg/kg bw per day for 6 days per week (equal to 0, 150, 300, 600, 1200 and 2400 mg/kg bw per day)	(No blood data) Vacuolization of hepatocytes	600	1200
Rat						
35 days subacute toxicity test	Oral	Calcium	0, 175, 350, 700, 1400 and 2800 mg/kg bw per day for 6 days per week (equal to 0, 150, 300, 600, 1200 and 2400 mg/kg bw per day)	Increase in serum albumin, glucose and AST levels, decrease of total cholesterol level, decreased absolute and relative weights of spleen and heart, increase of incident in vacuolated hepatocytes	150	300
182 days toxicity test	Oral	Calcium	0, 87.5, 175, 350, 700 and 1400 mg/kg bw fosfomycin per day for 6 days per week (equal to 0, 75, 150, 300, 600 and 1200 mg/kg bw per day)	Histopathological findings (vacuolated hepatocytes)	600	1200
Developmental toxicity	Oral	Calcium	0, 140, 700 and 1400 mg/kg bw per day f	Number of resorptions in early fetal stage and a delay in bone formation	700	1400
Two-generation study	IP	Sodium	0, 250, 750 and 1500 mg/kg bw per day f	Parental toxicity: reduction in spontaneous locomotor activity, excretion of loose stools, hepatic thickening and changes in other abdominal organs (secondary to the route of administration) Reproductive and offspring toxicity: 1500 mg/kg bw per day, the highest dose tested	750 (parental), 1500 (reproductive and offspring)	
Rabbit						
Developmental toxicity	Oral	Calcium	0, 80, 140 and 420 mg/kg bw per day f	–	420 (highest dose tested)	
Developmental toxicity	IV	Sodium	0, 80, 100, 200, 400 and 800 mg/kg bw per day f	Maternal: the highest dose tested Embryo/fetal: reduction in the body weight	800 (maternal) 400 (embryo/fetal)	

Table 6 (continued)

Species/study type	Route of administration	Fosfomycin salt	Doses	Critical end-point	NOAEL (mg/kg bw per day)	LOAEL (mg/kg bw per day)
Dog						
182 days subacute	Oral	Calcium	0, 280 and 560 mg/kg bw per day for 6 days per week (equal to 0, 240 and 480 mg/kg bw per day)	Liver thickening, renal congestion, swelling of tubular epithelium		240*

ADI: acceptable daily intake; AST: aspartate aminotransferase; BW: body weight; f: female; IP: intraperitoneally; IV: intravenous; LOAEL: least-observed-adverse-effect level; m: male; NOAEL: no-observed-adverse-effect level.
* Pivotal study for the derivation of the ADI *(16)*.

for interspecies and intraspecies variability, and because no NOAEL could be identified in the study. Given that the Committee was unable to assess the end-point of microbial resistance, it was not possible to determine an overall mADI. The Committee was therefore unable to establish an ADI for fosfomycin.

The Committee agreed that it was not necessary to determine a toxicological acute reference dose (ARfD) for fosfomycin in view of its low acute oral toxicity, and the absence of developmental toxicity and any other toxicological effects that would be likely to be elicited by a single dose. The Committee concluded that the microbiological effects (specifically, disruption of the intestinal colonization barrier) of fosfomycin should be used to characterize its acute risk, and therefore established an ARfD of 0.08 mg/kg bw.

A toxicological monograph was prepared.

Additional information that would assist in further evaluation of fosfomycin is information on the selection for and emergence of resistance in the microbiota in the gastrointestinal tract.

Appendix 1 – Details of the literature search

The Committee conducted a comprehensive review of the scientific literature from the following publicly accessible databases: Agricola, Embase, Web of Science, PubMed, Springer Nature Experiment, Food Science and Technology Abstracts, CABI VetMed Resource. The following searches were conducted in each of these databases:

- fosfomycin AND chicken/poultry/pig/swine AND residue
- fosfomycin AND chicken/poultry/pig/swine AND kinetics
- fosfomycin AND chicken/poultry/pig/swine AND withdrawal

Table A1
Inclusion and exclusion criteria for filtering articles retrieved in the literature search

Inclusion criteria	Exclusion criteria
Any article focusing on: • fosfomycin concentrations in plasma of chicken/pigs • fosfomycin concentrations in edible tissues of chicken/pigs • residue determination in chicken/pig tissues • bioavailability of fosfomycin residues in chicken/pigs No restrictions concerning year of publication	Any article focusing on: • bacteria resistance to fosfomycin • fosfomycin use in food animal species other than chicken/pigs • environmental issues • kinetics/residues of antimicrobials other than fosfomycin (and do not include fosfomycin for comparison) • in vitro effects of fosfomycin on bacteria • pharmacodynamics and efficacy of fosfomycin Articles already provided by the sponsor

The search strategy for literature relevant for the microbiological evaluation used the keywords "fosfomycin", "microbiome", "intestinal microbiota", "gut microbiota", "gut microbiome", "gastrointestinal microbiota", "gastrointestinal microbiome", "antimicrobial resistance", "susceptibility testing", "fosfomycin metabolism", "excretion" and "bioavailability" as well as the genus/species and minimum inhibitory concentration values of specific intestinal bacteria with the Boolean operators (AND, OR and NOT).

- fosfomycin AND chicken/poultry/pig/swine AND metabolism
- fosfomycin AND chicken/poultry/pig/swine AND analytical method.

Each search was conducted separately for each of the four species terms (i.e. 20 searches in total).

The criteria applied to filter the articles with regard to the assessment to be conducted are shown in Table A1.

Appendix 2 – Details of short-term studies of toxicity

Rat

Fosfomycin sodium was administered intraperitoneally to rats once daily for 35 days (except on Sundays) at doses of 125, 250, 500, 1000 and 2000 mg/kg bw *(65)*. There were three deaths in males in the 2000 mg/kg group and five deaths in females in the 2000 mg/kg group. The clinical signs observed were adoption of a stretching position and squeaking, which were considered to be due to local irritation caused by the administration route. Necropsy showed adhesion between intra-abdominal organs. Macroscopically, fibroplasia and adhesion of the liver capsule, ileum and caecal serosa were observed, but there were no other marked changes. With respect to mean body weight, there were no major differences between the control group and males in the 500 mg/kg group or females in the 1000 mg/kg group; however, there was a trend of slower weight gain compared

with the control group in males receiving 1000 mg/kg bw or more and females in the 2000 mg/kg group, with a corresponding decrease in food intake.

Blood biochemistry showed increased inorganic phosphate in males in groups that received 250 mg/kg or more, decreased blood urea nitrogen in males in the 250 mg/kg group and males in the groups that received 1000 mg/kg bw or more, and decreased sodium in males in the 125–500 mg/kg groups. There was decreased haemoglobin and increased chloride in females in the groups that received 500 mg/kg bw or more, and decreased white blood cells in females in all groups; however, both optical microscopy findings and comparison with background data found no specific organ abnormalities. In addition, effects were marginal and not consistent below 500 mg/kg bw.

In males, brains and hearts showed sporadic weight decrease, but there was no correlation with dose. The absolute weights of brains in the 1000 mg/kg group and of brains, lungs and testes (left and right) in the 2000 mg/kg group were lower than in the control group, while the relative ratio of organ weight to body weight was higher than in the control group. In females, kidneys (left and right) in the groups that received 250 mg/kg bw or more showed weight increase, adrenal glands (left) in the groups that received 1000 mg/kg or more showed weight increase, and livers in the groups that received 2000 mg/kg or more showed weight increase.

Corresponding to the necropsy findings in the males and females in the high-dose groups, there was fibroplasia or adhesion of the liver capsule, ileum and caecal serosa, but no other marked changes. Males and females in the high-dose groups had adhesion of the liver or hypertrophy at the edge of the liver, distension and digestive malabsorption of the ileum and caecum, and hypertrophy and adhesion of the intestinal wall. However, there were no marked changes in the other organs.

Fosfomycin sodium was administered intraperitoneally to rats (Wistar rats weighing 100 ±10 g and aged 5 weeks) for 182 days (except Sundays). There were no deaths in the males or females in the groups that received 500 mg/kg or less, three males and five females in the 1000 mg/kg group died, and all the animals in the 2000 mg/kg group died. The general condition changes observed were the same as those in our subacute toxicity study in rats. With respect to mean body weight, there were no major differences between the control group and males in the groups that received 500 mg/kg or less and females in the groups that received 1000 mg/kg or less. However, the groups that received a dose higher than these showed a trend of slowed weight gain compared with the control group. Mean food intake was similar to the control group in males and females in the groups that received 1000 mg/kg or less, but was lower than the control group in the 2000 mg/kg group, where all animals died. Blood tests showed changes in total protein, albumin, alkaline phosphatase, chloride and inorganic phosphate

in all dose groups and other parameters in higher dose groups. However, these changes were marginal compared to background, lacked dose dependency and did not change in a consistent direction; also, because optical microscopy did not show any specific organ abnormalities, they were not considered toxicological relevant. There was also mild degeneration of hepatocytes in males and females in the groups that received 500 mg/kg or more. Other findings included pneumonia with abscesses, inflammation around abdominal organs, and chronic infection of the spleen in males and females in the groups that received 500 mg/kg or more, but these effects are thought to be due to the irritation caused by the intraperitoneal administration, or increased susceptibility to infection resulting from large doses *(66)*.

Rabbit

Fosfomycin sodium was intravenously administered via the auricular vein to rabbits (0, 125, 250, 500, 1000 and 2000 mg/kg bw per day) once daily for 35 days (not on Sundays). There were no marked changes in general condition in any of the groups, and mean body weight and mean food intake were similar to the control group. Neither blood tests nor histopathological examination showed any abnormal findings caused specifically by fosfomycin sodium *(65)*.

Dog

Beagle dogs (female, weighing 8–12 kg) were administered fosfomycin sodium (100, 250 and 500 mg/kg for 91 days, except Sundays, two beagles in each group) *(66)*.

Compared with the control group, there were no marked changes with respect to the relative weight or absolute weight of any organs. There was mild cholestasis in part of the liver of one animal in the 500 mg/kg group. There were no other marked changes. There were small granulomatous nodules or infiltration of lymphocytes in the livers of two animals in the 250 mg/kg group and the 500 mg/kg group, as well as capillary dilation in one animal in the 500 mg/kg group. There were also small granulomatous nodules in the liver of one animal in the control group, and lymphocyte aggregation in the liver of another animal in that group. In the kidneys, there were small granulomatous nodules or infiltration of lymphocytes in two animals in the control group, two animals in the 100 mg/kg group, one animal in the 250 mg/kg group, and two animals in the 500 mg/kg group.

Beagle dogs (female, weighing 8–12 kg) were administered fosfomycin sodium (100, 250 and 500 mg/kg for 182 days, except Sundays, two beagles in each group) *(66)*.

Compared with the control group, there were no marked changes with respect to the relative or absolute weight of any organs. Although mild, there were scattered yellow granules in the liver of one animal in the 250 mg/kg group. There was also mild bleeding around blood vessels at the administration site in all groups, including the control group, but no other marked changes. There were scattered small granulomatous nodules in the liver of one animal in the 250 mg/kg group, and mild bleeding from the vascular wall or muscle tissue around the administration site in all groups, including the control group. There was also mild swelling of tubular epithelial cells in the 500 mg/kg group, but no other marked changes. In the liver, there were no marked changes in the 100 mg/kg group, but there was marked increase in organelles, particularly in the smooth endoplasmic reticulum (SER), in the 250 mg/kg group. Scattered between hepatocytes that looked almost normal or in small groups were cells that appeared bright all over; swollen cells with slightly condensed nuclei, dilated SER and rough endoplasmic reticulum (RER) lumens and markedly enlarged mitochondria; and cells that appeared dense, with changes such as condensed nuclei, swollen mitochondria in the cytoplasm and focal cytoplasmic degeneration. In addition, the Kupffer cells were enlarged, and phagocytosis was observed.

Intraperitoneal: perinatal and lactation period

In this study, fosfomycin sodium was administered intraperitoneally to adult Wistar rats during peri- and post-natal periods and examined for postnatal development and factors such as the reproductive ability of their offspring *(67)*. The fosfomycin sodium dosages used were 250, 750 and 1500 mg/kg, dissolved in physiological saline, with the pH adjusted to 7.0 with hydrogen chloride for a final concentration of 10% or 20%. The drug was administered intraperitoneally once a day on a regular basis. The administration period was from 14 gestation days (GD) to 21 days postpartum. In addition, the control group received physiological saline.

There was no significant effect of the drug on maternal (F0) body weight gain, but in the 1500 mg/kg group, suppression of spontaneous locomotor activity, excretion of loose stools and one dead fetus on the expected delivery date were observed. In the case of the dead fetus, a necropsy indicated abdominal organ adhesion and fetal residuals in the uterus, and the cause of death was presumed to be a fault in delivery. Additionally, the delivery rate of live offspring was significantly lower in the 750 and 1500 mg/kg groups than in the control group, but it appears that all groups had a frequent occurrence of perinatal dead fetuses. In the 1500 mg/kg group, uterine fetal residues were observed in necropsies of three maternal females. Necropsies of maternal females at the nursing stage dosed with 750 mg/kg or less indicated no obvious abnormalities; however, in

almost all cases of the 1500 mg/kg group, hepatic thickening, opaque membranes and adhesion of abdominal organs were observed.

In newborn offspring in the 1500 mg/kg group, a decrease in nursing rate was noted as well as a slowing of body weight gain. Additionally, in the 250 mg/kg group, delays were observed in the separation of auriculae, appearance of abdominal hair and vaginal opening compared with the control. However, this appeared to be due to the higher number of offspring per parent in this group than in the control group, and was not necessarily caused by the administration of fosfomycin sodium. There were no abnormalities in either group, as evidenced by the audiovisual test performed on day 28 after delivery or the conditioned avoidance test conducted on day 35. Furthermore, no abnormalities were observed at necropsies of animals on days 28 or 35 after delivery.

There was no effect of the drug on the reproductive ability of F1 and no specific influence on F2 was observed except for significantly lower fetal body weights in animals dosed at 750 mg/kg or more compared with the control. The decrease in fetal body weight appeared to be due to the higher number of fetuses per pregnant female in the 750 mg/kg group. The sex ratio of F2 was significantly different between the 250 mg/kg group and the control; however, the ratio in the control was somewhat low. Also, one of five pregnant females in the control group had a biased male:female fetus ratio of 3:12; in contrast, in two of six cases in the 250 mg/kg group, the ratio was biased 9:1 and 8:0. Thus, due to the dispersion of data, the differences in sex ratio were not considered to be toxicologically significant.

From the above, when fosfomycin sodium was administered to Wistar rats in their perinatal and postnatal periods, doses of 750 mg/kg or higher significantly lowered the delivery rates owing to perinatal mortality. Except in the group with the highest dose administered (1500 mg/kg) there were no specific effects on postnatal development, differentiation or behaviour, indicating that the drug has no effect on reproductive ability. The NOAEL for parental toxicity was 750 mg/kg bw per day based on suppression of spontaneous locomotor activity, excretion of loose stools, hepatic thickening and changes in other abdominal organs at 1500 mg/kg bw per day. The NOAEL for offspring toxicity was 1500 mg/kg bw per day, the highest dose tested. The NOAEL for reproductive effects was 1500 mg/kg bw per day, the highest dose tested.

Intravenous: organogenesis period

The young (F1) obtained on GD29 by performing caesarean sections of pregnant rabbits (New Zealand white, 14 weeks old, 10–15 animals per group) that received sodium fosfomycin by the intravenous route of administration (0, 80, 100, 200, 400 and 800 mg/kg bw per day) were examined from GD6 to GD18 *(62)*.

In dams (F0), no lethal cases or effects on body weight gain attributable to dose administration were observed. Only one case of fetal miscarriage was observed in the group treated at a rate of 400 mg/kg bw per day. Regarding the young (Fl), although low body weight was detected in females of the group treated at a rate of 800 mg/kg bw per day, no changes attributable to dose administration were observed in organ and bone malformation and mutation rates, fetal mortality rate, sex ratio in viable fetuses or the external appearance of fetuses. The NOAEL of this study was the highest dose (i.e. 800 mg/kg bw per day).

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Halquinol (addendum)

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1. Explanation

Halquinol (Chemical Abstracts Service [CAS] No. 8067-69-4) is a mixture of chlorinated products of quinolin-8-ol comprising 5,7-dichloroquinolin-8-ol (5,7-DCL or DCHQ; 57–74% weight per weight [w/w]), 5-chloroquinolin-8-ol (5-CL or CHQ; 23–40% w/w) and 7-chloroquinolin-8-ol (7-CL; 0–4% w/w).

Halquinol is a quinoline with broad-spectrum antimicrobial activity that acts by inhibiting respiratory enzymes in the cytoplasmic membrane of target organisms; this mode of action differs from that of quinolones. It is approved for use as a veterinary antimicrobial feed additive in multiple Member States. Halquinol is indicated for use in swine for the enhancement of feed efficiency, and for the control, treatment and prevention of scours or diarrhoea caused or complicated by *Escherichia coli* and *Salmonella* spp. It is administered orally at a feed inclusion rate of 60–600 mg/kg, for up to 10 consecutive days. Based on an estimated swine feed consumption of about 4% of body weight per day, the daily halquinol dose is about 2.4–24 mg/kg body weight (bw). Withdrawal periods

for approved halquinol products vary from 0 to 7 days. Halquinol has also seen limited use in humans.

Halquinol was evaluated at the 85th Joint Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA) meeting, in 2017 *(1)*. Its toxicology and microbiology, pharmacokinetics and metabolism in multiple species, and radiolabelled and non-radiolabelled studies in the target species (pigs) were evaluated. The sponsor proposed a marker residue (MR) for halquinol that comprised the sum of 5-CL and 5,7-DCL, and their glucuronide metabolites (expressed as 5-CL and 5,7-DCL equivalents).

At the 85th meeting, the Committee concluded that a toxicological acceptable daily intake (ADI) could not be established owing to the lack of information required to assess the in vivo mutagenicity and carcinogenic potential of halquinol. Furthermore, the Committee had numerous concerns with the residue depletion data provided – specifically, the limited extractability of radiolabelled residues and generally low total radioactivity in swine liver and kidney. Reliable estimates of the MR to total radioactive residue (TRR) ratios were not possible because of the significant uncertainties surrounding the liver and kidney radioactive residues. The Committee also noted that many of the extractable and non-extractable residues in these tissues were not fully characterized, and therefore could not accept the MR for halquinol proposed by the sponsor.

Non-radiolabelled halquinol residue depletion data were provided and were considered acceptable for deriving 95/95 upper tolerance limits (UTLs), based on the MR proposed by the sponsor, in muscle, liver, kidney and skin+fat. However, without reliable estimates for MR:TRR ratios, the total halquinol residues in tissues could not be estimated.

In 2017, the Committee concluded that "MRLs [maximum residue limits] could not be recommended for halquinol due to the lack of an established HBGV [health-based guidance value], incomplete characterization of residues in tissues (particularly liver and kidney), and a lack of data necessary to establish reliable MR:TRR ratios over time for calculation of total residues in tissues. The suitability of the proposed marker residue for halquinol cannot be confirmed without further characterization of the residues" *(1)*.

The present evaluation was conducted at the request of the Codex Committee on Residues of Veterinary Drugs in Foods (CCRVDF) as a follow-up evaluation. Although additional new data on in vivo mutagenicity or carcinogenicity were not submitted by the sponsor, the results of a comet assay in rats in vivo were submitted. The present Committee evaluated halquinol based on its previous assessment and on published data available.

2. Biological data

2.1 Biochemical aspects

Plasma levels of 5,7-dichloroquinolin-8-ol and 5-chloroquinolin-8-ol were measured in the 4-week and 13-week rat and minipig toxicity studies and the 13-week and 39-week dog toxicity studies. Other kinetic information was obtained using read-across from structurally similar compounds such as chloro-7-iodoquinolin-8-ol and 5,7-dibromoquinolin-8-ol in rats, guinea-pigs and rabbits. These oral pharmacokinetic studies showed that absorption of halogenated quinolin-8-ol compounds from the gastrointestinal tract was in the range of 30–40% in laboratory animal species. Following absorption, these compounds undergo extensive first-pass phase II metabolism to sulfate and glucuronide conjugates *(1)*.

In vitro incubation of ^{14}C-labelled 5,7-dichloro-8-quinolinol with isolated hepatocytes prepared from rats, dogs, minipigs, swine and humans showed that the two major metabolites were glucose and glucuronide conjugates. In hepatic microsomes, the major phase I metabolite common to all the microsome samples was hydroxy-5,7-dichloro-8-quinolinol *(1)*.

The predominant conjugate (sulfate or glucuronide) found in urine or bile/faeces differed among species. In rats, about 26% of a 15 mg/kg bw oral dose of radiolabelled 5,7-DCL and 5-CL (ratio 79:21) was excreted in urine while most of the remainder (69%) was detected in faeces less than 48 hours after dosing. In contrast, 80.5% was excreted in the urine of a ruminant (calf) administered an oral dose of 3 mg/kg bw by stomach intubation. Only about 10.5% of the radioactive dose was found in faeces, and the overall excretion rate was slower, with the radiolabel found in urine over 5–6 days. There was no evidence of bioaccumulation of radiolabelled compounds in the liver, kidney, muscle or fat *(1)*.

2.2 Toxicological studies

2.2.1 Acute toxicity study

The acute toxicity of halquinol has been investigated in mice and rats. The oral median lethal dose (LD_{50}) was 470–850 mg/kg bw in mice and 700 mg/kg bw in rats. In a more recent study, there were no deaths in rats after oral administration of a single dose at 500, 1000 or 2000 mg/kg bw, and no clinical signs at 500 or 1000 mg/kg bw. At 2000 mg/kg bw, one out of the three males showed piloerection 2 hours after the single-dose treatment *(1)*.

2.2.2 Short-term toxicity studies

(a) Rat

In a 28-day oral toxicity study in rats that was not good laboratory practice (GLP) compliant, halquinol was administered by gavage at doses of 0, 150, 450 or 1000 mg/kg bw per day. The no-observed-adverse-effect level (NOAEL) was 150 mg/kg bw per day based on histopathological changes in the liver (focal necrosis, congestion and hydropic degeneration) and kidney (cystic dilation of collecting tubules and necrosis of tubular epithelial lining) associated with a significant increase in clinical parameters *(1)*.

In a 4-week oral toxicity study in rats, halquinol was administered by gavage at doses of 0, 150, 450 or 750 mg/kg bw per day. The lowest-observed-adverse-effect level (LOAEL) was 150 mg/kg bw per day based on green faeces, changes in appearance and colour and traces of blood and crystals in urine, and histopathological changes in kidney and lymph nodes *(1)*.

In a 13-week oral toxicity study in rats with a 4-week recovery period, halquinol was administered daily for 13 weeks by gavage to male and female Sprague-Dawley rats at the dose levels of 50, 150 and 450 mg/kg per day in 0.5% methylcellulose. The NOAEL was 50 mg/kg bw per day based on increase in kidney weights (absolute and relative) associated with histopathological changes *(1)*.

(b) Pig

Three groups of three male and three female Göttingen minipigs received halquinol by oral gavage at dose levels of 25, 75 or 225 mg/kg per day with a dosage volume of 5 mL/kg per day for 4 weeks. At 225 mg/kg per day, one male was killed prematurely on Day 9 for humane reasons (numerous signs of toxicity were observed from Day 7). This animal lost weight and displayed disturbances of clinical pathology parameters. The gastrointestinal changes, red foci in the stomach and duodenum, white masses in the stomach, and liquid green content in the cecum and colon might have been related to the administration of halquinol. No histopathology was carried out. Apart from the above-mentioned animal, other clinical signs were minor and observed at low incidence: one male had a thin appearance, correlating with body weight loss recorded in the two surviving males at this dose level. There were increases in adrenal and decreases in thymus weights at 225 mg/kg per day attributed to stress in males. In the absence of microscopic examination, a relationship to halquinol treatment of organ weight changes could not be excluded for the kidneys and liver at 225 mg/kg per day in males and for the kidney at 75 mg/kg per day in males. There were no organ weight changes attributed to halquinol treatment in females. Macroscopically, the only finding potentially related to the test item at termination was tan and

enlarged kidneys in one male given the test item at 225 mg/kg per day. In view of the results obtained in this study, despite the absence of histopathology, the dose level of 75 mg/kg per day could be considered as the NOAEL in males and 225 mg/kg per day the NOAEL in females *(2)*.

In a planned 90-day study, halquinol was administered daily by oral administration (gavage) to male Göttingen minipigs at dose levels of 0, 25, 75 or 225 mg/kg per day (six animals for 0 or 225 mg/kg bw per day, four animals for 25 or 75 mg/kg bw per day). Based on mortality and severe signs of toxicity in the highest dose group, treatment of all animals was stopped on Day 3 (the last day of dosing was Day 2). Treatment of females was not initiated. At 225 mg/kg bw per day, two animals were found dead (one on Day 3 and one on Day 4), and a further two animals were terminated prematurely on Day 4 on humane grounds. Treatment-related clinical signs were observed with appreciable incidences at the high dose level (liquid or greenish faeces, hypoactivity, hypotonia, soft or liquid faeces, hypothermia and coldness to the touch). At 75 mg/kg bw per day, one animal had greenish or liquid faeces on Day 2. In the two decedent animals, there was evidence of disturbances of clinical pathology parameters (increased urea and creatinine levels, and increased liver enzymes, creatine kinase and lactate dehydrogenase activities). At histopathology, marked or severe acute tubular degeneration or necrosis of the kidneys was observed in the four minipigs that died or were killed prematurely in the highest dose group. One of these animals also had moderate single cell necrosis or apoptosis of the liver *(3, 4)*.

(c) Dog

In two toxicity studies in dogs, halquinol was administered in gelatin capsules at doses of 0, 3, 10, 60 or 150 mg/kg bw per day for 13 weeks, and at 0, 30, 60 or 90 mg/kg bw per day for 39 weeks. An overall NOAEL of 30 mg/kg bw per day was identified based on lower terminal body weights at 60 mg/kg bw per day in males *(1)*.

2.2.3 Long-term studies of toxicity and carcinogenicity

(a) Rat

In a 1-year chronic toxicity study in rats, halquinol was administered by oral gavage at 15, 50 or 150 mg/kg bw per day. The NOAEL was 15 mg/kg bw per day based on histopathological changes in kidney associated with an increase in mean absolute and relative kidney weights in females at 50 mg/kg bw per day *(1)*. In this study, there was no evidence for neoplastic progression in the kidney, and no preneoplastic lesions or carcinogenic precursors were identified in other tissues. Based on these results, halquinol may be unlikely to be genotoxic in vivo and unlikely to pose a carcinogenic risk to humans from the diet.

(b) Dog

A total of 32 beagle dogs (16 males and 16 females) were allocated to four groups and received halquinol BP80 in capsules at dose levels of 30, 60 and 90 mg/kg per day for 13 weeks. A fourth group acted as a control, and received an empty capsule per day. There were no deaths during the course of the study. In females at 90 mg/kg per day in Week 12, an increase in cholesterol (+33%) was observed. Adverse electrocardiogram (ECG) morphological abnormalities were observed in a single female at 90 mg/kg per day. The NOAEL was identified at 90 mg/kg per day in males and 60 mg/kg per day in females *(1)*.

(c) Carcinogenicity study

No new data on carcinogenicity were submitted by the sponsor.

2.2.4 Genotoxicity

Halquinol and its conjugates were tested in a range of in vitro tests, including bacterial reverse cell mutation tests, mammalian cell mutation tests using L5178Y TK+/– mouse lymphoma cells and chromosomal aberration tests in cultured lymphocytes, as shown in Table 1. 5,7-Dichloro-8-hydroxy-quinoline was negative in the Ames test using TA98 and TA100 *(5)*. Sulfates and glucuronides of metabolites were also negative in the Ames test *(6-9)*. However, halquinol tested positive in mouse lymphoma test for gene mutations and in human lymphocytes test for chromosome aberration in vitro *(1)*.

Two adequate studies conducted in vivo, examining the potential of halquinol to induce chromosome damage in rat bone marrow cells, were negative *(1)*. In these studies, four groups of five male and five female Sprague-Dawley rats received a single oral administration of halquinol at the dose levels of 500, 1000 and 2000 mg/kg bw. No increase in the frequency of micronucleated cells was noted in females at any of the tested dose levels, at either of the sampling times. Halquinol did not induce damage to the chromosomes or the mitotic apparatus of rat bone marrow cells after a single oral administration at the dose levels of 500, 1000 and 2000 mg/kg bw.

The positive result in the in vitro mammalian mutagenicity study was not addressed by the in vivo studies (micronucleus and chromosome aberration – both of which were performed using bone marrow), because the latter did not assess the induction of a gene mutation. Moreover, structure–activity relationship (SAR) analysis indicated the presence of structural alerts for both mutagenicity and clastogenicity for all three components of halquinol *(1)*.

The sponsor newly submitted the results of a comet assay in rats in vivo. Halquinol was evaluated for its genotoxic potential using the comet assay to assess DNA damage in liver and jejunum cells of male rats (TG489), as shown in Table 2.

Table 1
Summary of mutagenicity studies of halquinol

Study type	Test system	Concentration / dose tested	Results	Reference
Ames test	*Salmonella typhimurium* TA98, TA100	5,7-dichloro-8-hydroxyquinoline at 0.05 g/mL (w/wo S9)	Negative (w/wo)	*(5)*
Miniscreen Ames test	*Salmonella typhimurium* TA98, TA100	5-chloro-8-hydroxyquinoline sulfate 5-chloro-8-hydroxyquinoline glucuronide 5,7-dichloro-8-hydroxyquinoline sulfate 5,7-dichloro-8-hydroxyquinoline glucuronide At 5, 15, 50, 150, 500 µg/well (±S9)	Negative (±S9)	*(6-9)*
Gene mutations in mammalian cells in vitro	L5178Y TK+/– mouse lymphoma cells	0.31, 0.63, 1.25, 2.5, 5, 10, 20 and 40 µg/mL (w/wo S9; 40 µg/mL precipitated)	Positive	(w/wo) *(10)*
Chromosome aberration in vitro	Human lymphocytes	0, 0.78, 1.56, 3.13, 6.25, 12.5, 25, 50 and 100 µg/mL (wo S9) 0, 3.13, 6.25, 12.5, 18.8, 25, 37.5, 50 and 100 µg/mL (w S9)	Positive (w)	*(11)*
Micronucleus in vivo	Rat	500, 1000 and 2000 mg/kg per day (oral)	Negative	*(12)*
Chromosome aberration in vivo	Rat	500, 1000 and 2000 mg/kg per day (oral)	Negative	*(13)*
Comet assay in vivo	Rat	500, 1000 and 2000 mg/kg per day (oral)	Negative	*(14)*

w: with; wo: without.

Table 2
Percentage tail DNA in liver cells following administrations in the Comet assay

Treatment (10 mL/kg per dose)	Number of animals	Group mean % of clouds	Tail DNA (%) mean ± standard deviation
Vehicle control			
Methylcellulose (4000 cps) at 0.5% (w/v) in deionized water	6	0.5	0.15 ±0.08
Halquinol			
500 mg/kg per day	6	0.5	0.20 ±0.13
1000 mg/kg per day	6	0.3	0.15 ±0.07
2000 mg/kg per day	6	0.3	0.34 ±0.18*
Positive control			
Ethyl methanesulfonate 200 mg/kg	3	0.3	15.06 ±3.31**
Male rat historical control data 2008–2017			
Vehicle	0.00–4.35 (minimum and maximum) 0.98 (95% confidence interval)		

cps: centipoise; DNA: deoxyribonucleic acid; w/v: weight per volume.
* $P \leq 0.05$ (Student's t-test); statistically significant increase relative to the vehicle control.
** $P \leq 0.05$ (analysis of variance [ANOVA], Dunnett's post hoc); statistically significant increase relative to the vehicle control.

The definitive assay dose levels tested were 500, 1000 and 2000 mg/kg per dose. No statistically significant increases in percentage tail DNA were observed for any dose level in the jejunum. A statistically significant twofold increase in percentage tail

DNA was observed in the 2000 mg/kg per dose group in the liver; however, there was no difference at any other dose level and the increase was within the testing facility's historical control range for this organ (mean ± standard deviation [SD] 0.17 ±0.40; range 0.00–4.36, 95% confidence interval [CI]: 0.00–0.98). The authors concluded that halquinol was negative in vivo in the comet assay *(14)*. However, the Committee concluded that the moderate increase in 2000 mg/kg per dose group in the liver is a significant effect on DNA, and that it is possible that a high dose of halquinol may cause weak DNA damage, although the carcinogenic risk to humans from the diet may be negligible.

2.2.5 Reproductive and developmental toxicity

(a) Multigeneration reproductive toxicity

In a two-generation reproductive toxicity study in rats, halquinol was administered by oral gavage at doses of 0, 50, 150 or 450 mg/kg bw per day. The NOAEL for parental toxicity was 50 mg/kg bw per day based on increased kidney and spleen weights at 150 mg/kg bw per day in F0 and F1 parents. The NOAEL for reproductive toxicity was 450 mg/kg bw per day based on the absence of any treatment-related effects. The NOAEL for offspring toxicity was 150 mg/kg bw per day, based on histopathological kidney changes in pups at 450 mg/kg bw per day *(1)*.

(b) Developmental toxicity

In a developmental toxicity study in mice, halquinol was administered by oral gavage on gestation days (GDs) 6–17 at doses of 0, 30, 100 or 300 mg/kg bw per day. The NOAEL for maternal toxicity was 30 mg/kg bw per day based on clinical signs (round back, piloerection, pallor of extremities, emaciated appearance or hyperactivity/hypoactivity) at 100 mg/kg bw per day (Table 3). Based on these changes, the acute reference dose (ARfD) was also estimated as 0.3 mg/kg bw.

The NOAEL for embryo/fetal toxicity was 30 mg/kg bw per day, based on an increase in the incidence of delayed ossification at 100 mg/kg bw per day (Table 4). No evidence of teratogenicity was observed *(1)*.

In a developmental toxicity study in rats, halquinol was administered by oral gavage on gestation days 6–20 at doses of 0, 100, 300 or 1000 mg/kg bw per day. The NOAEL for maternal toxicity was 300 mg/kg bw per day based on clinical signs (e.g. ptyalism) observed at 1000 mg/kg bw per day. The NOAEL for embryo/fetal toxicity could not be established because of lower mean female fetal body weights at 100 mg/kg bw per day, the lowest dose tested. No evidence of teratogenicity was observed. The Committee concluded that halquinol was not teratogenic in mice and rats *(1)*.

Table 3
Summary of clinical signs in females during pregnancy

	Dose (mg/kg per day)			
	0	**30**	**100**	**300**
Mortality				
Found dead (after treatment)	0	0	1	0
Final sacrifice	24	24	22	23
Found dead	0	0	1	1
General aspect				
Round back	0	0	1	5
Piloerection	0	0	2	6
Convulsions	0	0	1	0
Emaciated appearance	0	0	1	0
Pallor of extremities	0	0	0	1
Behaviour				
Hypoactivity	0	0	1	1
Hyperactivity	0	0	3	4
Breathing				
Abdominal breathing	0	0	1	0
Secretion or excretion				
Reflux at dosing	0	1	1	0
Normal				
No remarkable observations	24	23	19	16

Table 4
Litter and fetal incidences of skeletal variations with statistical significance

	Dose level (mg/kg per day)				
	0	**30**	**100**	**300**	**HCD[a]**
Dams with live fetuses, *n*	16	21	17	14	111
Live fetuses, *n*	119	140	117	87	719
Head-skull					
Frontal: incomplete ossification, L(F) %	18.8 (4.2)	9.5 (2.1)	41.2 (11.1)	64.3* (25.3#)	29.6 (6.9)[a]
Supraoccipital: incomplete ossification, L(F) %	12.5 (1.7)	9.5 (2.1)	23.5 (8.5*)	71.4** (29.9#)	20.8 (4.0)[a]
Parietal: incomplete ossification, L(F) %	0 (0)	9.5 (2.9)	11.8 (4.3*)	50.0** (13.8#)	8.3 (1.3)[a]
Supraoccipital: bipartite ossification, L(F) %	6.3 (0.8)	0 (0)	5.9 (1.7)	21.4 (6.9*)	0 (0)
Nasal: incomplete ossification, L(F) %	0 (0)	0 (0)	0 (0)	14.3 (4.6*)	8.3 (1.3)[a]
Cervical vertebrae					
Incomplete ossification of centrum, L(F) %	0 (0)	4.8 (0.7)	5.9 (0.9)	28.6* (6.9**)	10.0 (3.3)[a]
Unossified centrum, L(F) %	0 (0)	4.8 (0.7)	0 (0)	28.6* (10.3#)	20.8 (4.0)[a]
Caudal vertebrae					
Unossified centrum, L(F) %	6.3 (0.8)	4.8 (0.7)	11.8 (5.1)	21.4 (13.8#)	45.8 (11.9)[a]
Incomplete ossification of centrum, L(F) %	12.5 (1.7)	9.5 (1.4)	11.8 (4.3)	35.7 (17.2#)	20.0 (6.9)[a]

Table 4 (continued)

	Dose level (mg/kg per day)				
	0	30	100	300	HCD[a]
Sternebrae					
Bipartite ossification, L(F) %	0 (0)	9.5 (1.4)	0 (0)	21.4 (5.7*)	40.0 (6.3)[a]
Incomplete ossification of 6th, L(F) %	6.3 (0.8)	23.8 (4.3)	17.6 (4.3)	57.1** (20.7#)	25.0 (8.6)[a]
Incomplete ossification of 1st to 4th, L(F) %	6.3 (0.8)	14.3 (2.9)	47.1* (9.4**)	35.7 (14.9#)	16.7 (4.0)[a]
Unossified 5th, L(F) %	0 (0)	0 (0)	5.9 (0.9)	14.3 (5.7*)	8.3 (1.3)[a]
Metacarpal bones					
Incomplete ossification of metacarpals, L(F) %	0 (0)	0 (0)	11.8 (4.3*)	21.4 (11.5#)	12.5 (3.3)[a]
Forepaw phalanxes					
Unossified distal phalanx, L(F) %	0 (0)	0 (0)	5.9 (1.7)	7.1 (4.6*)	5.0 (1.5)[a]
Unossified proximal phalanx, L(F) %	0 (0)	0 (0)	5.9 (5.1*)	14.3 (11.5#)	12.5 (3.3)[a]
Hindlimb					
Unossified talus, L(F) %	100 (63.9)	95.2 (70.7)	100 (83.8#)	100 (96.6#)	100 (86.7)[a]
Unossified calcaneus, L(F) %	31.3 (9.2)	38.1 (13.6)	47.1 (18.8*)	85.7** (54.0#)	63.0 (31.8)[a]
Metatarsal bones					
Incomplete ossification of metatarsals, L(F) %	0 (0)	0 (0)	5.9 (5.1*)	28.6* (6.9**)	16.7 (5.3)[a]
Unossified 1st, L(F) %	0 (0)	0 (0)	0 (0)	7.1 (8.0**)	0 (0)
Hindpaw phalanxes					
Unossified distal phalanx, L(F) %	6.3 (0.8)	0 (0)	5.9 (1.7)	7.1 (8.0*)	8.3 (1.3)[a]
Incomplete ossification proximal phalanx, L(F) %	6.3 (0.8)	14.3 (2.1)	17.6 (2.6)	50.0* (19.5#)	14.8 (3.5)
Unossified proximal phalanx, L(F) %	0 (0)	9.5 (1.4)	11.8 (6.8**)	35.7* (14.9#)	0 (0)

F: fetal; HCD: historical control data; L: litter.
Statistical significance: *: $P<0.05$; **: $P<0.01$; #: $P<0.001$. Underlined results indicate treated litter percentage higher than the upper limit of the HCD.
[a] HCD were from Citoxlab France: effects on embryo/fetal development in mice; five studies from February 2006 to November 2008; (a) denotes maximum percentage.

Halquinol was administered by gavage once daily from days 6 to 20 post coitum inclusive, to pregnant Sprague-Dawley rats at dose levels of 50, 150 or 350 mg/kg per day in a dose-range finding study. The control group was administered the vehicle, methylcellulose at 0.5% (w/w) in drinking-water treated by reverse osmosis, under the same experimental conditions. On the basis of the results obtained in this study, the dose level of 350 mg/kg per day was well tolerated in pregnant rats. There were no adverse effects on embryo/fetal development *(15)*.

2.3 Microbiological effects

The Committee used a decision-tree approach to determine the need to establish a microbiological acceptable daily intake (mADI) for halquinol. The decision-tree approach, which complies with International Cooperation on Harmonization

of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) guidelines *(16)*, was adopted by the 66th JECFA meeting *(17)*.

The decision-tree approach first determines if microbiologically active residues are entering the human colon. If no microbiologically active residues are entering the colon, no mADI is necessary. If microbiologically active residues are present in the human colon, two end-points of public health concern must be considered: 1) disruption of the colonization barrier; and 2) increase of the population(s) of resistant bacteria. At Step 4 of the decision-tree process, it is possible to provide scientific justification to eliminate testing (i.e. the need for an mADI) for either one or both end-points. Step 5 is where an mADI is determined. Should an mADI not be necessary, the toxicological or pharmacological ADI is used.

The Committee evaluated minimum inhibitory concentration (MIC) susceptibility testing data, microbiological activity studies of halquinol and its metabolites, antimicrobial resistance studies and animal studies to determine bioavailability for use in the decision-tree to answer the following questions in the assessment of halquinol.

Step 1: Are residues of the drug, and (or) its metabolites, microbiologically active against representatives of the human intestinal flora?

Yes. Halquinol is a broad-spectrum antimicrobial agent that is microbiologically active against a wide variety of Gram-negative and Gram-positive bacteria including genera and species representative of the human intestinal microbiota. Halquinol is also active against fungi and protozoa. Halquinol metabolites have very reduced or no measurable activity against intestinal microbiota.

In a GLP-compliant study, Pridmore (2015) *(18)* determined the MICs of halquinol and each of the metabolites 5-chloro-8-hydroxyquinoline sulfate, 5-chloro-8-hydroxyquinoline β-D-glucuronide, 5,7-dichloro-8-hydroxyquinoline sulfate and 5,7-dichloro-8-hydroxyquinoline β-D-glucuronide against 90 bacterial strains, comprising 10 isolates from each of nine groups of genera representing the normal human intestinal microbiota: *Bifidobacterium*, Gram-positive non-spore forming rods (*Eubacterium* group), *Clostridium*, *Bacteroides* species, Gram-positive anaerobic cocci (*Peptostreptococcus* group), *Fusobacterium*, *Enterococcus*, *E. coli* and *Lactobacillus*. All bacterial strains were obtained from the faeces of healthy unmedicated human volunteers. The test system was standardized agar dilution MIC methodology using quality control strains as described in the Clinical and Laboratory Standards Institute guidelines *(19, 20)*. Each MIC was determined using inoculum levels at 10^6 to 10^8 colony-forming units (CFU)/mL, depending upon the strain tested.

The activities of halquinol and its metabolites against each bacterial group are summarized in Table 5. MIC_{50}, MIC_{90} and geometric mean were calculated for each bacterial group. Halquinol exerted greatest activity against *E. coli* (MIC_{50} of 16 µg/mL). *Bifidobacterium* species was the least susceptible (MIC_{50} of 256 µg/mL). Halquinol activity was also clearly demonstrable against Gram-positive anaerobic *cocci* (*Peptostreptococcus* group) *Fusobacterium*, *Enterococcus* and *Bacteroides*, all with MIC_{50} values of 32 µg/mL. *Lactobacillus* and Gram-positive anaerobic rods (*Eubacterium* group) had MIC_{50} of 64 µg/mL.

In general, none of the halquinol metabolites showed antibacterial activity, having MIC values greater than 256 µg/mL (Table 5) *(18)*.

A study investigating the effect of halquinol on caecal microbiota in broiler chickens, using DNA sequencing and metagenomics techniques, found no significant changes in the composition of the caecal microbiota *(21)*.

Step 2: Do residues enter the human colon?

Yes. Pharmacokinetic studies in experimental models (humans, rats, guinea-pigs, rabbits and beagle dogs) indicated that halquinol after oral administration is rapidly metabolized and excreted in the urine, mostly as sulfate and glucuronide conjugates, with little absorption in the gastrointestinal tract (see Section 2.1.1). A study in humans using structurally similar analogues determined that up to 25% of the dose is excreted in the urine over 72 hours *(22)*. Another study in male rats indicated that about 30% of halquinol and metabolites were excreted in the urine *(23)*. A study with pigs using ^{14}C-labelled halquinol indicated that halquinol is rapidly excreted, mainly via urine, with recovery of radioactivity in the faeces averaging about 36% *(24)*. Based on its similarity to the urinary excretion of halquinol analogues by humans, rat urinary excretion of halquinol determined by Bories & Tulliez (1972) *(23)* was used to estimate the fraction of the oral dose available to microorganisms.

Halquinol residues could enter the colon of a person ingesting tissues from treated food-producing animals.

Step 3: Do the residues entering the human colon remain microbiologically active?

Yes. Halquinol residues are microbiologically active. However, halquinol is extensively metabolized by many animal species and humans. Sulfation and glucuronidation of 5-CL and 5,7-DCL appear to be the major metabolic pathways. Glucose conjugates and oxidative de-chlorination metabolites have also been reported. The MIC_{50} values of the sulfate and glucuronide conjugates of 5-CL and 5,7-DCL were greater than 256 µg/mL, suggesting that their antibacterial activities are low or almost nonexistent compared with halquinol (see Table 5). Nevertheless, since intestinal bacteria have hydrolytic enzymes such as

Table 5

Microbiological activity of halquinol and its metabolites against bacterial strains representing human intestinal microbiota

Test item	Summary MIC parameter	Value (µg/ml) for each bacterial group								
		Enterococcus	*Escherichia coli*	*Clostridium*	*Bacteroides*	*Bifidobacterium*	*Lactobacillus*	*Fusobacterium*	Gram-positive anaerobic cocci	Gram-positive non-sporing anaerobic rods
Halquinol	Range	8 - 32	8 - 16	All 32	32 - 64	32 - 256	32 - 64	16 - 32	4 - 64	32 – 64
	MIC_{50}	32	16	32	32	256	64	32	32	64
	MIC_{90}	32	16	32	64	256	64	32	64	64
	Geo. mean	26	12	32	37	181	60	24	34	56
5-chloro-8-hydroxyquinoline sulfate	Range	128 - >256	All >256	All >256	All >256	All >256	All >256	All >256	256 - >256	All >256
	MIC_{50}	>256	>256	>256	>256	>256	>256	>256	>256	>256
	MIC_{90}	>256	>256	>256	>256	>256	>256	>256	>256	>256
	Geo. mean	>239	>256	>256	>256	>256	>256	>256	>256	>256
5-chloro-8-hydroxyquinoline β-D-glucuronide	Range	All >256	All >256	All >256	All >256	All >256	All >256	All >256	All >256	All >256
	MIC_{50}	>256	>256	>256	>256	>256	>256	>256	>256	>256
	MIC_{90}	>256	>256	>256	>256	>256	>256	>256	>256	>256
	Geo. mean	>256	>256	>256	>256	>256	>256	>256	>256	>256
5,7-dichloro-8-hydroxyquinoline sulfate	Range	64 - >256	All >256	All >256	All >256	128 - >256	All >256	All >256	64 to >256	128 - >256
	MIC_{50}	>256	>256	>256	>256	256	>256	>256	>256	>256
	MIC_{90}	>256	>256	>256	>256	256	>256	>256	>256	>256
	Geo. mean	>223	>256	>256	>256	>239	>256	>256	>194	>239
5,7-dichloro-8-hydroxyquinoline β-D-glucuronide	Range	All >256	All >256	All >256	All >256	All >256	All >256	All >256	All >256	All >256
	MIC_{50}	>256	>256	>256	>256	>256	>256	>256	>256	>256
	MIC_{90}	>256	>256	>256	>256	>256	>256	>256	>256	>256
	Geo. mean	>256	>256	>256	>256	>256	>256	>256	>256	>256

Pridmore A (2015)
Source: Pridmore (2015) *(18)*.

β-glucuronidase and arylsulfatase, there is potential for the halquinol metabolites to be deconjugated back to the parent compound in the gastrointestinal tract.

Step 4: Is there any scientific justification to eliminate testing for either one or both end-points of concern – i.e. disruption of the colonization barrier or resistance development?

Halquinol does not appear to induce or select for the development of resistance in bacteria. Results from a study in which halquinol was fed to pigs over a 6-week period did not induce resistance to *E. coli (24)*. In addition, although halquinol has been in widespread use in human and veterinary medicine, there have been no microbiological studies suggesting the development of resistance to halquinol. Therefore, the only end-point of concern is the disruption of the colonization barrier. Consequently, there is a need to determine an mADI for halquinol residues.

Step 5: Derivation of an mADI using the VICH GL36 guideline approach

The formula for deriving the upper bound of the mADI for the end-point of concern, disruption of the colonization barrier, is as follows:

$$\text{mADI} = \frac{\text{MIC}_{\text{calc}} \times \text{Mass of colon content}}{\text{Fraction of oral dose available to microorganisms} \times \text{Body weight}}$$

where:

- MIC_{calc} is derived from the lower 90% confidence limit for the mean MIC_{50} of the relevant genera for which the drug is active (as described in Appendix C of VICH GL36 *(25)*). The MIC_{calc} is derived as follows:

$$\frac{\text{Std dev}}{\text{Lower 90\% CL}} = \text{Mean MIC}_{50} - \sqrt{n} \times t_{0.10,\,\text{df}}$$

where:

- Mean MIC_{50} is the mean of the log-transformed MIC_{50} values;
- Std dev is the standard deviation of the log-transformed MIC_{50} values;
- n is the number of MIC_{50} values used in the calculations; and
- $t_{0.10,\text{df}}$ is the 90th percentile from a central t-distribution with degrees of freedom (df) = $n-1$.

Mass of colon volume: The Committee adopted the 500 mL value – based on the volume measured in humans by Pritchard et al. (2013) *(26)* – for use in the evaluation instead of 220 g.

Fraction of oral dose available to microorganisms: It is recommended that this value be based on in vivo measurements for the drug administered

orally. Alternatively, if sufficient data are available, this value can be calculated as 1 minus the fraction of an oral dose excreted in urine. The fraction may be lowered if quantitative in vitro or in vivo data show that the drug is inactivated during transit through the intestine. Human data are encouraged, but if none are available, non-ruminant animal data are recommended. In the absence of data to the contrary, it should be assumed that metabolites have antimicrobial activity equal to the parent compound.

Based on similar rates of excretion in rats and humans – about 30% in rats, according to Bories & Tulliez (1972) *(23)* and 25% in humans treated with structurally related analogues, according to Berggren & Hansson (1968) *(22)* – the fraction of oral dose of halquinol available in the colon is 1 – 0.30 = 0.70.

Body weight: An adult human is assumed to weigh 60 kg.

Therefore, the upper bound of the mADI for halquinol is calculated as follows:

$$\text{mADI} = \frac{24 \text{ mg/mL} \times 500 \text{ mL}}{0.70 \times 60 \text{ kg bw}} = \textbf{0.0285 mg/kg bw per day}$$

The MIC_{calc} of 24.0395 µg/mL is based on the MIC_{50} for *E. coli* (16 µg/mL), *Bifidobacterium* (256 µg/mL), *Clostridium* (32 µg/mL), *Bacteroides* (32 µg/mL), *Lactobacillus* (64 µg/mL), *Fusobacterium* (32 µg/mL), *Eubacterium* (32 µg/mL) and *Peptostreptococcus* (32 µg/mL) (Table 5).

The microbiological acute reference dose (mARfD) for halquinol was determined using the following formula:

$$\text{mARfD} = \frac{(MIC_{calc} \text{ or NOAEC}) \times \text{Correction factors} \times \text{Colon volume}}{\text{Fraction of oral dose available to microorganisms} \times \text{Body weight}}$$

$$= \frac{24 \text{ mg/mL} \times 3 \times 500 \text{ mL}}{0.70 \times 60 \text{ kg}} = \textbf{0.0857 mg/kg}$$

For the effects on the colonization barrier, an mADI of 0.857 mg/kg bw (60 kg) was established.

2.4 Observations in humans

No reliable information on toxicological effects of halquinol in humans was available *(1)*.

Some toxicity case reports are reported as observations in humans by sponsor.

The patient, aged 42 years, had worked for 29 years in an animal feed mill as a foreman. Two weeks before the onset of a rash he had been assisting in

adding "Quixalaud" to the feeds. A contact dermatitis developed on the backs of the hands and wrists. The rash cleared up when he stopped work and did not recur when he went back to work that did not involve contact with Quixalaud. A patch test with 1% Quixalaud (halquinol 60% and chalk 40%) gave a marked positive reaction *(27)*.

In Saudi Arabia in 1975, a patient aged 40 years with diarrhoea had taken Squibb's "Quixalin" (halquinol) tablets as directed on the packet. Some 2 weeks after beginning to take the tablets, she noticed tingling sensations and then numbness in her feet, which in the ensuing months spread up the legs to the hips, and resulted in difficulty in walking. The legs later showed partial improvement. Her vision deteriorated and then she became blind for practical purposes *(28)*.

3. Comments

3.1 Biochemical aspects

No new studies on the pharmacokinetics of halquinol in laboratory species were submitted. Previously evaluated studies had demonstrated that absorption of halogenated quinolin-8-ol compounds from the gastrointestinal tract in laboratory species was in the range 30–40%. Following absorption, the compounds undergo extensive first-pass metabolism to the sulfate and glucuronide conjugates. The major metabolite produced on incubation of hepatic microsomal fractions with ^{14}C-5,7-DCL was hydroxy-5,7-DCL *(1, 29, 30)*.

In rats, the major route of excretion of the conjugates was in the faeces, whereas in a calf it was in the urine. There was no evidence of bioaccumulation of radiolabelled compounds in liver, kidneys, muscle or fat *(1)*.

3.2 Toxicological data

The toxicological target of halquinol was the kidney in rats and dogs; cytotoxic damage to renal tubules was observed *(1)*. Continuous cytotoxic damage to the kidney suggested carcinogenic potential of halquinol on chronic exposure. However, although renal toxicity was observed in a 1-year chronic toxicity test in rats, there was no evidence for neoplastic progression in the kidney, and no preneoplastic lesions or carcinogenic precursors were identified in other tissues.

Halquinol was positive in a mouse lymphoma cell assay and a chromosomal aberration test in cultured lymphocytes in vitro. However, it showed no evidence of genotoxicity in Ames tests in vitro, or in tests in vivo for

chromosomal aberrations in bone marrow or micronuclei in bone marrow *(1)*. No DNA damage was observed in liver or jejunum in the newly submitted comet assay.

The Committee concluded that halquinol is unlikely to be genotoxic in vivo.

The Committee concluded that halquinol is unlikely to pose a carcinogenic risk to humans from the diet, given that it is unlikely to be genotoxic in vivo and any carcinogenicity would be secondary to prolonged preneoplastic damage, for which there was no indication on chronic (1 year) exposure of rats.

3.3 Microbiological data

No new studies were submitted on microbiological activity of halquinol and its metabolites against bacterial strains representative of the human intestinal microbiota and antimicrobial resistance. Therefore, the mADI of 0.29 mg/kg bw and mARfD of 0.86 mg/kg bw values determined by the 85th JECFA meeting in 2017 *(1)* were used by the Committee to compare with the toxicological HBGVs.

4. Evaluation

The Committee determined a toxicological ADI for halquinol of 0–0.15 mg/kg bw on the basis of a NOAEL of 15 mg/kg bw per day for histopathological changes in the kidney, accompanied by increases in absolute and relative renal weight in a 1-year chronic toxicity study in rats, with application of a safety factor of 100 to account for interspecies and intraspecies variability. The Committee concluded that the toxicological effects of halquinol were the most relevant for characterizing its chronic risk, and therefore established the ADI as 0–0.2 mg/kg bw (rounded to 1 significant figure).

The Committee determined a toxicological ARfD for halquinol of 0.3 mg/kg bw on the basis of a NOAEL of 30 mg/kg bw for clinical signs in dams observed in a developmental toxicity study in mice, with application of a safety factor of 100 to account for interspecies and intraspecies variability. The Committee concluded that the toxicological effects of halquinol were the most relevant for characterizing its acute risk, and therefore established the ARfD as 0.3 mg/kg bw.

Studies relevant to the risk assessment are summarized in Table 6.

Table 6
Summary of toxicity studies – halquinol

Species/study type (route of administration)	Doses (mg/kg bw per day)	Critical end-point	NOAEL (mg/kg bw per day)	LOAEL (mg/kg bw per day)
Mouse				
Developmental study (gavage)	0, 30, 100, 300	Maternal toxicity: clinical signs	30**	100
		Embryo and fetal toxicity: delayed bone ossification	30	100
Rat				
13-week toxicity (gavage)	0, 50, 150, 450	Histopathological lesions in the kidneys	50	150
1-year toxicity and carcinogenicity study (diet)	0, 15, 50, 150	Histopathological lesions in the kidneys	15*	50
Two-generation reproduction study (gavage)	0, 50, 150, 450	Parental toxicity: increase in kidney and spleen weights	50	150
		Offspring toxicity: kidney lesions in pups	150	450
		Reproductive toxicity: none	450[a]	–
Developmental study (gavage)	0, 100, 300, 1000	Maternal toxicity: clinical signs	300	1000
		Embryo and fetal toxicity: lower mean fetal body weights correlating with delayed bone ossification	–	100[b]
Dog				
13-week toxicity study (gelatin capsule)	0, 3, 10, 60, 150	Body weight loss	30[c]	60[d]
39-week toxicity study (gelatin capsule)	0, 30, 60, 90	Body weight loss		

ADI: acceptable daily intake; ARfD: acute reference dose; bw: body weight; LOAEL: lowest-observed-adverse-effect level; NOAEL: no-observed-adverse-effect level.
* Pivotal study value used for the establishment of the ADI.
** Pivotal study value used for the establishment of the ARfD.
[a] Highest dose tested.
[b] Lowest dose tested.
[c] Overall NOAEL.
[d] Overall LOAEL.

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Selamectin

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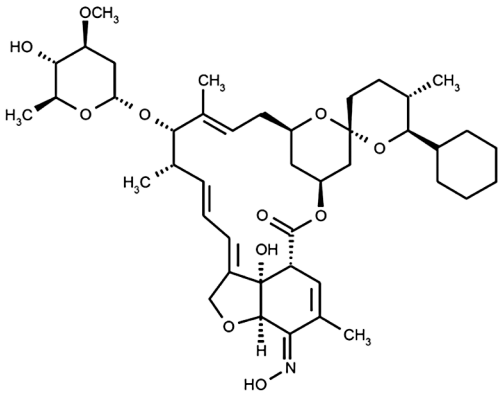
1. Explanation

Selamectin (International Union of Pure and Applied Chemistry [IUPAC] name (2aE,4E,5'S,6S,6'S,7S,8E,11R,13R,15S,17aR,20Z,20aR,20bS)-6'-cyclohexyl-3',4',5',6,6',7,10,11,14,15,17a,20,20a,20b-tetradecahydro-20b-hydroxy-20-hydroxyimino-5',6,8,19-tetramethyl-17-oxospiro[11,15-methano-2H,13H,17Hfuro[4,3,2-pq] [2,6]benzodioxacyclooctadecin-13,2'-[2H]pyran]-7-yl2,6-dideoxy-3-O-methyl-a-L-arabino-hexopyranoside; Chemical Abstracts Service [CAS] No. 165108–07–6) is a semisynthetic macrocyclic lactone compound of the avermectin class, a large family of broad-spectrum parasiticides, and is widely used as an endectocide against nematode and arthropod parasites in dogs and cats.

Selamectin is not currently approved for use in food-producing animals. However, the Joint Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA) evaluated selamectin at the present meeting as part of a pilot programme in which it conducts a parallel review of the information at the same time as the sponsor pursues approval in the proposed species with national authorities, as discussed at the 24th session of the Codex Committee on Residues of Veterinary

Drugs in Foods (CCRVDF) *(1)*. The Committee was asked to establish relevant health-based guidance values (HBGVs) and recommend maximum residue limits (MRLs) in Atlantic salmon.



Chemical structure of selamectin

Selamectin is under development for the control of sea lice infestations in Atlantic salmon. It is intended as a 7-day, in-feed ectoparasiticide additive for treatment and prevention of all parasitic stages of sea lice on Atlantic salmon, ranging from smolts to market weight fish, in seawater. The product is to be administered in feed to fish at an appropriate feeding rate for 7 days to yield a dose rate of selamectin of 100 µg/kg biomass per day. A withdrawal period has not been proposed.

Selamectin, like other avermectins, acts mainly on a glutamate-gated chloride channel (GluCl) that is present in both neuronal and muscle membranes of invertebrates, but is not present in vertebrates. Normally, avermectins are also agonists of gamma aminobutyric acid chloride channels (GABACls) in the central nervous system (CNS) of invertebrates and vertebrates; however, the binding affinity of selamectin in the mammalian brain is 100-fold lower than the affinity for binding sites in invertebrates *(2)*. In addition, selamectin has a much lower binding affinity to the GABACls in the vertebrate CNS than ivermectin *(3)*.

The Committee reviewed a data package submitted by a sponsor. Most studies contained certificates of compliance with good laboratory practice (GLP), including all of the critical studies. Additionally, the following databases of published literature were searched using the search term "selamectin" (with the number of retrieved articles shown in parenthesis): Agricola (1), Aquaculture Compendium (0), Agris (264), Analytical Abstracts (5), ASFA (0), CAB Abstracts (296), CAS (714), Embase (354), FSTA (2), Oceanic Abstracts (0), Reaxis (596), Scopus (1112) and WOS (380). In total, 3724 articles were retrieved, of which

1983 were removed as duplicates. The titles and abstracts of the remaining 1741 articles were screened to determine their relevance. Information from articles considered relevant was included in this assessment.

2. Biological data

2.1 Biochemical aspects

2.1.1 Absorption, distribution and excretion

(a) Oral and intravenous administration

(i) Rats

In a non-GLP-compliant study, Sprague Dawley rats (5/sex) were orally administered (oral dosing method not specified) with a single dose of 6 mg/kg body weight (bw) of selamectin (purity and solvent not specified) or were intravenously administered selamectin at 1 mg/kg bw. Plasma samples were collected at 5 minutes (intravenously treated animals only) and at 1, 2, 4, 7, 24, 48 and 72 hours postdose. One male and one female were randomly assigned to necropsy at 1, 2, 4 and 72 hours. In relation to oral administration, this study verified that the peak concentration in plasma (C_{max}) of males and females presented similar mean values (1190 ng/mL and 1260 ng/mL, respectively). The area under the plasma concentration–time curve from time 0 to infinity ($AUC_{0-\infty}$), however, was 2.5-fold greater in males than in females (18 600 and 46 200 ng/hour per mL, respectively). Elimination half-life ($t_{1/2}$) also differed between males and females (7.6 hours and 22.0 hours, respectively). The oral bioavailability in males was 58.5% and in females was 43.7%. In relation to intravenous administration, as in the oral study, there was a marked difference between male and female rats in most pharmacokinetic parameters, with females showing higher concentrations and longer half-life times. The $AUC_{0-\infty}$ following intravenous administration was 5.33 µg/hour per mL in males and 17.7 µg/hour per mL in females, with elimination $t_{1/2}$ of 7.74 hours in males and 24.1 hours in females. Selamectin concentrations in the brain paralleled those in plasma, with a brain to plasma ratio of about 13% *(4)*.

In another non-GLP-compliant study, Sprague Dawley rats (number of animals and gender not specified) received, by oral gavage, a single dose of 10 mg/kg bw of selamectin (purity not specified) in sesame oil. Blood samples were collected at 0 (pre-dose) and 2, 4, 6, 24, 48 and 72 hours postdose. Selamectin was rapidly absorbed with an average C_{max} of 1.30 ±0.22 µg/mL and maximum plasma concentrations (T_{max}) at 4 ±1 hour. The elimination half-life was 10.3 hours. The area under the plasma concentration–time curve from time zero to time of last

measurable concentration (AUC_{last}) was 22 ±4 µg/hour per mL and the mean $AUC_{0-\infty}$ was 23 ±4 µg/hour per mL. No distinction was made between males and females for this study *(5)*.

In a non-GLP-compliant study, Sprague Dawley rats (3 male and 5 female) received by intravenous bolus a single dose of 0.1 mg/kg bw of selamectin (purity not specified) in glycerol formal, benzyl alcohol solution HS15 and NaCl 0.9% glucose. Blood samples were collected at pre-dose (0), 5, 10 and 15 minutes, and at 1, 2, 6, 10, 24 and 48 hours postdose. There was a marked difference in the overall exposure of selamectin in plasma between males and females. The plasma concentration of selamectin was below the limit of quantitation in male rats after 2 hours but was detected in most female rats for up to 24 hours postdose. The $AUC_{0-\infty}$ was 865 ±165 ng/hour per mL in female rats. The volume of distribution (V_{ss}) in female rats was 2.23 ±0.37 L/kg. Clearance (CL) was 115.67 ±22.03 mL/kg per hour, and half-life was about 16.5 hours. The AUC, volume of distribution, clearance and half-life of elimination could not be determined accurately in male rats owing to limited points on the elimination phase of the curve *(6)*.

The concentration of selamectin in plasma was investigated in all repeated-dose toxicity studies in rats.

In a non-GLP-compliant study, selamectin (97.3% purity) was administered in the diet to Sprague Dawley rats (4/sex per dose) at a dose of 0, 15, 40 or 80 mg/kg bw per day for 7 days (actual dose of 0, 4.3, 13.4, 35.4 and 70.1 mg/kg bw per day for males and 0, 4.5, 12.6, 34.7 and 70.1 mg/kg bw per day for females), or 14 days (actual doses of 4.5, 13.5, 34.5 or 69.5 mg/kg bw per day for males and 4.5, 12.6, 34.7 and 70.0 mg/kg bw per day for females). Blood samples were taken at 0, 3 and 11 hours postdose on day 7 and day 14. The time to reach T_{max} of selamectin was 0 hours in both sexes across the tested dose levels on days 7 and 14, except on day 14 in the 4.5 mg/kg bw per day female group, where the T_{max} was observed to be 3 hours. The C_{max} of selamectin ranged from 362 to 22 000 ng/mL at all dose levels in both sexes on day 7. On day 14, the C_{max} of selamectin ranged from 520 to 21 950 ng/mL at all dose levels in both sexes. The AUC_{last} showed a more than dose-dependent increase, from 5 to 80 mg/kg bw per day in both sexes on days 7 and 14. The male to female exposure (AUC_{last}) ratios ranged from 0.12 to 0.57 across the tested dose levels on days 7 and 14; based on the exposure profile, this finding suggested a sex-related difference across the dose levels, where females had twofold to eightfold higher exposure than males. No accumulation was noted at the tested dose levels *(7)*.

In a GLP-compliant study, selamectin (99.6% purity) was administered for 28 days in the diet to Sprague Dawley rats (4/sex per dose). Males received selamectin at doses of 0, 15, 40 or 80 mg/kg bw per day (actual dose of 0, 14.6, 41.2 or 75.9 mg/kg bw per day), or females received selamectin at doses of 0, 5, 15 or 40 mg/kg bw per day (actual dose of 0, 4.5, 15.0 or 40.9 mg/kg bw per

day for females). Blood samples were collected on days 15 and 27, at 6 am (0 hours postdose), 9 am (3 hours postdose) and 5 pm (11 hours postdose). Mean plasma concentrations revealed that selamectin concentrations were similar for each dose level and time point throughout the day. In addition, overall, there was little increase in plasma concentrations from days 15 to 27. Also, on day 27, at the 15 mg/kg per day dose level there was a 3.8-fold increase in selamectin plasma concentrations in the females compared with the males, and at the 40 mg/kg per day dose level there was a 2.3-fold increase in selamectin plasma concentrations in the females compared with the males *(8)*.

In a 3-month toxicity GLP-compliant study, selamectin (purity 86.6%, in sesame oil) was administered to Sprague Dawley rats (5/sex per dose) by oral gavage at doses of 0, 5, 15 and 80 mg/kg bw per day (male), and 0, 5, 15 and 40 mg/kg bw per day (female). Blood samples were collected at 1, 3, 8 and 24 hours postdose on days 13, 30 and 90. The mean plasma drug concentrations increased with increasing dose, and were about twice as high at most time points in female rats as in male rats when normalized for dose. The AUCs were similar on days 13 and 30 within the same sex, but were generally higher on day 90 than on day 13 *(9)*.

In a GLP-compliant study, selamectin (97.9% purity) was administered for 13 weeks in the diet to Sprague Dawley rats (4/sex per dose). Males received selamectin at doses of 0, 15, 40 or 80 mg/kg bw per day (actual doses of 0, 14.6, 39.1 or 75.4 mg/kg bw per day) and females received selamectin at doses of 0, 1, 15 or 40 (actual doses of 0, 0.8, 15.9 or 39.4 mg/kg bw per day). Blood samples were collected on days 15, 30, 45 or 91, at 6 am (0 hours postdose), 9 am (3 hours postdose) and 5 pm (11 hours postdose). Results indicated that exposure to selamectin increased with increasing dose in a reasonably dose-proportional manner for both males and females. At the dose of 15 mg/kg bw per day, females had about 1.6 times to 4.2 times higher exposure (C_{max}) than males; at 40 mg/kg bw per day, females showed a greater exposure (2.8 to 4.2 times) than males *(10)*.

In a 1-year GLP-compliant study, selamectin (98.7% purity) was administered in the diet to Sprague Dawley rats (6/sex per dose). Males received doses of 1, 5, 15 or 40 mg/kg bw per day and females received doses of 0.3, 1, 5 or 15 mg/kg bw per day. Blood samples were collected on days 1, 29, 92, 183, 274 and 365 postdose. Exposure increased in a more than dose-proportional manner at all sample times. Selamectin was readily absorbed from the diet but with some variability among the animals, with the increases becoming closer to proportional over time. Substantial accumulation was evident, with accumulation ratios based on the AUC between times 0 and 24 hours (AUC_{0-24h}) ranging from 8.3 to 11.8 in females and 3.54 to 8.81 in males. By the end of the study, female and male rats in the highest dose groups (15 mg/kg bw per day and 40 mg/kg bw per day, respectively) were exposed to mean concentrations of 19.0 µg/mL and

11.3 µg/mL, respectively. As expected, females had substantially more exposure than males at the same dose. Accumulation of selamectin was evident for both males and females in all dose groups. Females accumulated more selamectin than males, but accumulation was less in the highest dose groups in both males and females. Steady state appeared to have been achieved between 274 and 365 days *(11)*.

In a maternal toxicity GLP-compliant study, pregnant Sprague Dawley rats (5/dose) were dosed by oral gavage with doses of 0, 10, 40 or 60 mg/kg bw per day of selamectin (purity not specified), from gestation day (GD) 6 to postnatal day (PND) 10. At PND8–10 (5 hours postdose), plasma concentrations were 2.5, 16.2 and 21 µg/mL for 10, 40 and 60 mg/kg, respectively, and milk concentrations were 1.8, 20 and 19 µg/mL, respectively *(12)*.

In a non-GLP-compliant dose range-finding prenatal developmental study, pregnant Sprague Dawley rats (6/dose) were dosed by oral gavage with 0, 20, 32, 50 or 80 mg/kg bw per day of selamectin (99.6% purity) in 1.0% carboxymethylcellulose (CMC) and 0.5% Tween 80, from GD6 to 19. Blood samples were collected from three rats per group at 0 (pre-dose), 1, 2, 4, 8 and 24 hours after dose administration on GD19. All surviving toxicokinetic phase animals were euthanized on GD20, and a terminal blood sample was collected. The GD19 plasma drug levels of selamectin increased in a near proportional manner as the dose increased from 20 to 32 to 50 mg/kg bw per day. However, at the 80 mg/kg bw per day dosage level, the GD19 plasma drug levels were often lower than those observed in the 50 and 32 mg/kg bw per day dosage groups. The director of the study suggested that this decrease in selamectin plasma levels at the high dosage may be due to an intestinal toxicity (lymphatic vasodilatation) at the highest dose, which prevented drug absorption. The fetal plasma levels increased as the dosage increased in the 80 mg/kg bw per day dosage group. However, the fetal plasma levels were just a small fraction of those observed in the dams *(13)*.

In a GLP-compliant dosage range-finding multigeneration toxicity study, selamectin (99.6% purity) was administered in the diet to pregnant Sprague Dawley rats (4/dose) at doses of 0, 5, 10, 25 or 50 mg/kg bw per day (actual doses of 0, 5.3, 10.5, 27.3 or 51.5 mg/kg bw per day during GD0–18, and 5.9, 12.3, 31.4 or 61.2 mg/kg bw per day during lactation day (LD) 0–21 respectively). Blood samples were collected on PND4, 14 and 21 from both dams and pups (1 pup per litter). Plasma concentrations in dams and pups generally increased with increasing dose levels of selamectin. At day 5 after birth, differences between male and female pups in plasma concentration were minimal, but by day 15 and day 22 females showed a higher selamectin concentration *(14)*.

In another GLP-compliant multigeneration toxicity study, selamectin (99.6% purity) was administered in the diet to Sprague Dawley rats (25/sex/group) at doses of 0, 5, 15 or 50 mg/kg bw per day. Dosing began 70 days prior

to mating. Selamectin was administered to offspring selected to become the F1 parental generation following weaning (i.e. on PND 21). The F0 and F1 males continued to receive the test and control diets throughout mating until the day of euthanasia. The F0 and F1 females continued to receive the control and test diets throughout mating, gestation and lactation until the day of euthanasia. Blood samples for exposure assessment were collected from five F0 rats per sex per group on day 65, from five F1 rats per sex per group on PND 80, and from five F1 and F2 litters per group (2 culled pups/sex per litter) on PND 4. Exposure of selamectin was confirmed in F0 dams and in F1 and F2 pups. Concentrations were twofold to threefold higher in F0 and F1 females compared with males on day 65 and PND 80, respectively. However, selamectin concentrations in F1 and F2 pups on PND 4 were similar between the sexes. Therefore, adult females had higher exposure than males at all exposure levels. Regardless, exposures increased with dose in a nearly dose-proportional manner for both sexes *(15)*.

In a GLP-compliant oral developmental neurotoxicity study, selamectin (96.3% purity) was administered in the diet to Sprague Dawley rats (25 females/group) at doses of 0, 5, 15 or 50 mg/kg bw per day. Pregnant dams received selamectin from GD6 to PND22. F1 generation pups were directly exposed to the test and/or control substances towards the end of the pre-weaning period when they began consuming food (~PND15 to 22). Pups may also have been exposed to the test and/or carrier control substances during gestation (in utero exposure) or via maternal milk during the lactation period. Blood samples were collected from mothers on PND22 and from pups (F1 generation) on PND5. Exposure of selamectin was confirmed in dams and pups. Plasma concentrations in dams and pups generally increased with increasing doses of selamectin. The selamectin concentration in pups was 20–35% of that seen in the dams *(16)*.

(ii) Rabbits

In a non-GLP-compliant oral dose range-finding developmental toxicity study, groups of New Zealand rabbits (6 females/group) were given selamectin (99.6% purity) in 1.0% CMC and 0.5% Tween 80 by oral gavage at doses of 0, 20, 32 or 80 mg/kg bw per day from GD7 to GD28. Blood samples for plasma analysis were collected from all females on GD29. Following maternal blood collection and euthanasia, blood samples were collected from one viable fetus from each litter. Mean fetal plasma concentrations were about 30–50% that of the maternal values; thus, fetal plasma concentration as a percentage of dam plasma concentration was higher in the rabbit than the rat. The average rabbit fetal concentration ranged from 23.4% (at 32 mg/kg) to 48.2% (at 80 mg/kg) of the maternal values. Plasma concentrations increased approximately proportionally as the dose increased for both the dam and fetuses *(17)*.

In another non-GLP-compliant oral dose range-finding developmental toxicity study, groups of New Zealand rabbits (3 females/group) were dosed with selamectin (99.6% purity) in 1.0% CMC and 0.5% Tween 80 by oral gavage at doses of 0, 125, 250, 500 or 1000 mg/kg bw per day from GD7 to GD28. Maternal blood samples were collected from all females on GD7, 14, 21, 27 and 29. In addition, fetal blood samples (1 fetus/litter) were collected at the time of laparohysterectomy. Following the first dose, selamectin exposure increased with dose for both AUC_{0-24h} and C_{max} between 125 and 500 mg/kg bw per day, but there was no increase in exposure between 500 and 1000 mg/kg bw per day. The increase between 125 and 500 mg/kg bw per day was less than dose-proportional. Despite daily dosing, the pregnant rabbits had decreasing selamectin plasma concentrations at GD28. The fetal concentrations of selamectin were similar to those in dams *(18)*.

In a GLP-compliant prenatal developmental toxicity study, groups of New Zealand rabbits (4 females/group) were dosed by oral gavage with doses of 0, 20, 60 or 180 mg/kg bw per day with selamectin (99.6% purity) in 1.0% CMC and 0.5% Tween 80 from GD7 to GD28. Blood samples were collected from all females at 0 (pre-dose) and about 1, 2, 4, 8 and 24 hours postdose on GD28. After daily dosing for 22 days, C_{max} of selamectin in pregnant rabbits was 739, 1550 and 1390 ng/mL for the 20, 60 and 180 mg/kg bw per day dose groups, respectively. Time to peak ranged from 1–2 hours for the 20 mg/kg bw per day group, 4–24 hours for the 60 mg/kg bw per day group and 1–8 hours for the 180 mg/kg bw per day group. The AUC_{0-24h} and C_{max} increased with dose; however, the increase was less than dose-proportional *(19)*.

(iii) Dogs

In a GLP-compliant study, beagle dogs (6/sex) were dosed orally by gavage with a single dose of 24 mg/kg bw of the commercial formulation of selamectin (Revolution, 93.3/92.4% purity) in sesame oil. Blood samples were collected at 1, 2, 4, 7, 12, 18, 24, 32, 48, 72 and 96 hours postdose, and at 6, 8, 12, 16, 21, 28, 35 and 42 days postdose. The oral bioavailability in dogs was 62%. Following oral administration of selamectin at 24 mg/kg bw, the mean C_{max} was 7.63 µg/mL, which occurred at T_{max} of about 8 hours with a terminal half-life of 45.7 hours. Pharmacokinetic parameters appeared to be independent of sex *(20)*.

In a GLP-compliant study on intravenous administration, beagle dogs (6/sex per dose) received a single 30-minute intravenous infusion of the doses 0.05, 0.1 or 0.2 mg/kg bw of selamectin (93.3%/92.4% purity) in glycerol formal, benzyl alcohol, solutol HS15, 0.9% NaCl and 5% glucose. Blood samples were collected one day prior to each treatment; at 5, 10 and 15 minutes after starting the infusion; and at 5, 15 and 30 minutes and 1, 2, 4, 7, 12, 24, 48, 72, 96, 144

and 192 hours postdose. The C_{max} at the end of the infusion and the $AUC_{0-\infty}$ were linearly related to the dose level. The systemic body clearance was low (1.1 mL/kg per minute) and the steady state volume of distribution was moderate (1.2 L/kg). After completion of the infusions at each dose, plasma concentrations showed a biphasic decline, with a terminal half-life of 14.0 hours *(20)*.

(b) **Dermal application**

(i) Rats

In a non-GLP-compliant study, Sprague Dawley rats (5/group, sex not specified) received the commercial 6% formulation of selamectin (12 mg/kg bw), applied to a shaved dorsal surface. At 8, 12, 24, 36, 48, 60, 72, 96 and 120 hours postdose, rats were euthanized and blood was collected. Selamectin was slowly absorbed following topical application, resulting in low systemic exposure. The C_{max} was 38 ng/mL, with a T_{max} of 24 hours. The terminal half-life of elimination was 60 hours. The mean $AUC_{0-t(last)}$ was 2102 ng/hour per mL, and the mean $AUC_{0-\infty}$ was 2957 ng/hour per mL. Relative to oral dosing, topical bioavailability was about 8% *(21)*.

(ii) Rabbits

In a non-GLP-compliant study, New Zealand white rabbits (3/sex/group) infested with fleas (*Ctenocephalides felis*) received topically applied selamectin (formulation and purity not specified) at doses of 0, 10 or 20 mg/kg bw. Blood samples were collected from a central ear artery, cephalic vein or lateral saphenous vein at time 0 (immediately before topical administration) and at 6 and 12 hours, and 1, 2, 3, 5, 7, 14, 21 and 28 days after treatment. Selamectin was rapidly absorbed transdermally and was rapidly eliminated in rabbits. Mean terminal half-life, C_{max}, T_{max} and $AUC_{0-\infty}$ of selamectin were 0.93 days, 91.7 ng/mL, 0.45 days and 140.4 ng/hour per mL, respectively, for rabbits in the 10 mg/kg bw group; and 0.97 days, 304.2 ng/mL, 0.5 days and 432.0 ng/hour per mL, respectively, for rabbits in the 20 mg/kg group *(22)*.

(iii) Dogs

In a non-GLP-compliant study, beagle dogs (6/sex) received 24 mg/kg bw of the commercial formulation of selamectin (Revolution, 93.3/92.4% purity) in butylated hydroxytoluene plus dipropylene glycol monomethyl ether and isopropyl alcohol. The dose, divided into three equal volumes, was applied onto the skin of the animal's back at three different sites: base of the neck and scapulae, 15 cm posterior to the first application site and about 15 cm posterior to the second application site. Blood samples were collected one day prior to selamectin application, and at 4, 6, 9, 12, 18, 24, 32, 48, 56, 72, 80, 96, 104, 120, 144 and 166

hours postdose and 9, 11, 13, 16, 20, 24, 28, 32 and 38 days postdose. The dermal availability of selamectin was 4.4%. The C_{max} was about 86.5 ng/mL and was reached at about 3 days after selamectin application. Sex-related differences in C_{max} and AUC were not statistically significant. The half-life of elimination could not be estimated adequately because it did not meet the acceptance criteria *(20)*.

In a GLP-compliant study, the dermal absorption of [^{3}H]-selamectin (commercial product) in dipropylene glycol monomethyl butylhydroxytoluene was applied to beagle dogs (3/sex) at a single site on the skin of the animal's back at a dose level of 1 mL of a 12% formulation (120 mg/mL), providing a mean dose of about 12 mg/kg bw. Urine, faeces and pan-rinse specimens were collected on a daily basis, and animals were euthanized at 42 days postdose. Over the 42 days, 15.42–22.96% of the dose was recovered in faeces, 1.09–4.94% in urine, 16.59–28.88% in shed hair, 2.01–12.02% from the carcass and an additional 11.36–18.08% in cage rinses. The total percentage of dose recovered per animal ranged from 59.08% to 73.35% *(23)*.

2.1.2 Biotransformation

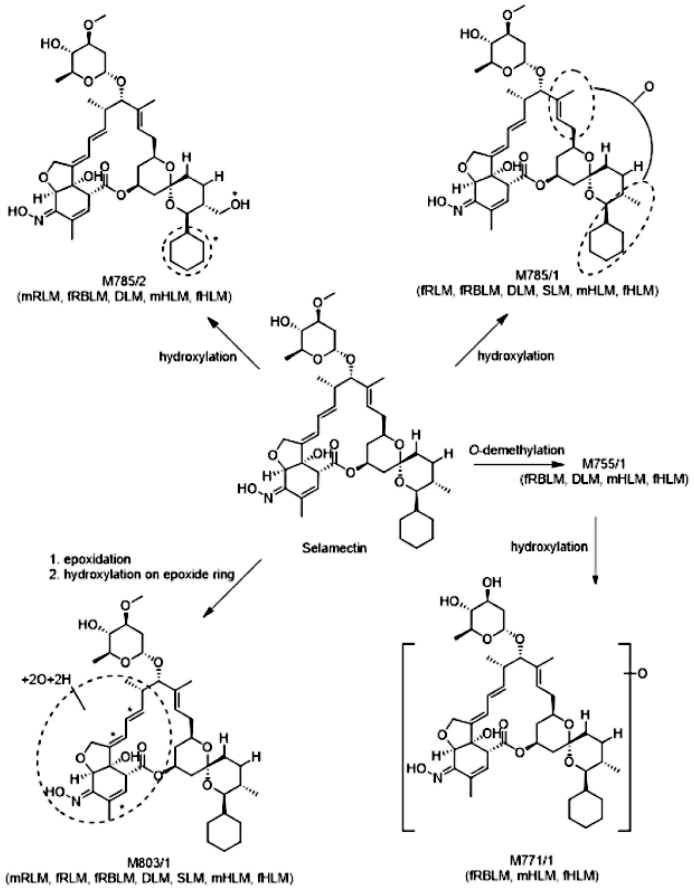
(a) In vivo

In a GLP-compliant study, the metabolism of [^{3}H]-selamectin in dipropylene glycol monomethyl butylhydroxytoluene was investigated in dogs (3/sex). Following a single topical dose of radiolabelled selamectin (commercial formulation: Revolution, 93.3/92.4% purity) on the skin of the animal's back at a dose level of 1 mL of a 12% formulation (120 mg/mL), selamectin was eliminated in the faeces (18–20% of the dose) and urine (1–3% of the dose) over 42 days. Most of the radioactivity in the faeces was associated with the parent compound (39% in females and 64% in males). Selamectin accounted for 99–100% of the total radioactivity observed in urine from male and female dogs, with no metabolites detected. Several oxidative O-desmethyl metabolites of selamectin, constituting less than 10% of the total radioactivity, were detected in dog faeces samples *(24)*. The metabolic pathway for selamectin is shown in Fig. 1.

(b) In vitro

An in vitro GLP-compliant comparative metabolism study was performed. The aims were to determine the metabolite profiles of [^{3}H]-selamectin in liver microsomes from Sprague Dawley rats (male and female), New Zealand white rabbits (female), beagle dogs (sex unspecified), Atlantic salmon (sex unspecified) and humans (male and female); and to characterize or identify the prominent metabolites of [^{3}H]-selamectin in the samples by liquid chromatography/mass spectrometry (LC/MS). [^{3}H]-selamectin at final nominal concentrations of 1 and 10 μM was incubated in duplicate with liver microsomes from male rats,

Fig. 1
Proposed metabolic pathway for selamectin



female rats, female rabbits, dogs, salmon, male humans and female humans in the presence of nicotinamide-adenine dinucleotide phosphate, reduced form (NADPH) for 0.5 and 1 hour. The samples were incubated in a water bath maintained at about 37 °C, except for the salmon liver microsomal samples, which were incubated at about 25 °C.

The unchanged selamectin accounted for more than 70% of the total radioactivity (TRA) at the end of the incubation in liver microsomes from male and female rats, female rabbits, dogs, salmon, and male and female humans. Among the five metabolites, M785/2 was the most abundant metabolite in male rats (3.31–3.88% of TRA), female rabbits (5.61–11.86% of TRA), male humans

(4.42–6.80% of TRA) and female humans (4.76–8.68% of TRA). M785/2 was not observed in liver microsomes from female rats and salmon. In dog liver microsomes, M755/1 was the most abundant metabolite; it accounted for 2.01–4.66% of TRA. M785/1 and M803/1 were the only two observed metabolites in liver microsomes from female rats and salmon. These results indicate that the metabolites produced by salmon are also produced by laboratory species (i.e rats and rabbits). The metabolites were derived from pathways involved in mono-oxidation in different positions, O-demethylation and/or epoxidation accompanying hydroxylation on the epoxide ring.

All metabolites observed in male and female human liver microsomes were present in the female rabbit liver microsomes. Gender-dependent selamectin metabolism was not observed in human liver microsomes but was seen in rat liver microsomes. There were no unique human metabolites: all metabolites observed in human liver microsomes were also observed in liver microsomes from at least one other animal species *(25)*.

2.2 Toxicological studies

2.2.1 Acute toxicity

(i) Mice and rats

In a GLP-compliant study, Nahas (1994) *(26)* investigated the acute oral toxicity of selamectin (94.7% purity) in CD-1 mice and Sprague Dawley rats (5/sex per dose for each species). Selamectin was administered in a single dose of sesame oil by oral gavage to mice and rats at doses of 800 or 1600 mg/kg bw in volumes of 10 or 20 mL/kg bw, respectively. Control animals received the vehicle at 20 mL/kg bw. All animals were observed for mortality and clinical or behavioural signs for the first 5 minutes postdose, then at 0.25, 0.5, 1, 2, 3 and 5 hours postdose, and daily thereafter for 14 days. Body weights were recorded on days 1, 7 and 14. All animals were euthanized and necropsied on day 14. No early deaths occurred in any of the mice or rats, and no treatment-related findings were noted at necropsy. Diarrhoea, which occurred from 15 minutes postdose, was observed in both mice and rats and at both dose levels. Rats that were administered 1600 mg/kg bw had additional clinical signs that included dyspnoea, hunched posture, and chromodacryorrhea in 9/10 rats; in general, these signs first appeared 3–24 hours postdose and lasted up to 24 hours postdose. However, in some animals, these signs appeared up to 76 hours postdose and were still present at up to 91 hours. Rats administered 1600 mg/kg bw also had partially closed eyes, decreased activity and piloerection (in 2–5 of 10 rats). These signs first appeared 3–95 hours postdose and lasted 1–96 hours. In mice, no clinical signs other than diarrhoea were observed.

In a GLP-compliant acute oral toxicity study that used the up-and-down procedure *(27)*, young Sprague Dawley rats (5 females) underwent a 16–20 hour feed-and-fast cycle before being administered, by oral gavage, the Selarma formulation (selamectin 12% fish). Initially, a single female rat was orally dosed with selamectin at a dose level of 2000 mg/kg bw. Because the animal survived, four additional females were dosed at 2000 mg/kg. All five female rats survived a single oral dose of 2000 mg/kg bw. The rats were observed for mortality, toxicity and pharmacological effects at 15 minutes, 1, 2 and 4 hours postdose, and twice daily thereafter for 14 days. Body weights were recorded pretest, weekly and at euthanasia. All animals were examined for gross pathology. No abnormal physical signs were observed among these five animals. Localized hair loss was observed on one animal for 5 days. All five animals gained weight by the end of the study. The gross necropsy revealed no observable abnormalities. The oral median lethal dose (LD_{50}) of selamectin 12% fish is greater than 2000 mg/kg bw in female rats *(28)*.

(ii) Dogs

In a GLP-compliant study, beagle dogs (6/sex) were dosed orally by gavage at a single dose of 24 mg/kg bw of the commercial formulation of selamectin (Revolution, 93.3%/92.4% purity) in sesame oil. Each animal served as its own control and was examined 7 days prior to exposure. On the day of dosing, detailed clinical observations were made prior to dosing, within 10 minutes after dosing, and 2, 4 and 8 hours after dosing by a veterinarian who was unaware of the treatment. Similar clinical observations were made once daily from day 1 to day 6 (inclusive) postdose. The dogs were monitored twice daily for general physical appearance and behaviour, food and water consumption and appearance of urine and faeces. There were no clinical signs or effects on body weight that were attributable to treatment following acute oral exposure *(20)*.

2.2.2 Short-term studies of toxicity

(i) Rats

In a non-GLP-compliant 14-day oral range-finding study, Sprague Dawley rats (5/sex/group) were administrated selamectin (purity: 93.3%, solvent: sesame oil) by oral gavage. Treated groups received 0, 80, 160 or 240 mg/kg bw per day for 14 days. In all groups, the rats were observed daily after treatment for mortality and clinical signs. Body weights were recorded –2 days before dosing and then on days 1, 3, 7, 10 and 14. Food consumption was measured over periods of 6 or 7 days on days 1–7 and 7–14, and water consumption was measured over periods of 24 hours on days 2–3 and 8–9. Haematology and clinical chemistry investigations were performed about 24 hours after the last treatment. At the

end of the study, rats were euthanized and necropsied, and several organs were weighed. Histopathological examinations were carried out on a range of tissues. Mortality, severe clinical signs, reduction in food intake and body weight, and gastrointestinal changes occurred at doses of 160 and 240 mg/kg bw per day in males and at all doses in females. Marked electrolyte imbalance and a decrease in concentration of plasma lipids occurred in both sexes and at all doses of selamectin. In addition, there were minor clinical signs, a fatty change in the liver and significant increases in liver enzyme and bilirubin at all doses; atrophy of adipose tissues at mid and high doses; cutaneous ulceration at the high dose; and haemorrhage in the adrenal cortex at low and high doses. Overall, the treatment produced toxic effects at all doses. However, at the low dose, toxicity was less severe than at the mid and high doses *(29)*.

In a non-GLP-compliant 14-day dietary dose range-finding toxicity study, selamectin (97.3% purity) was administered in the diet to Sprague Dawley rats (4/sex per dose) at a dose of 0, 15, 40 or 80 mg/kg bw per day for 7 days (actual doses of 0, 4.3, 13.4, 35.4 or 70.1 mg/bw per day for males, and 0, 4.5, 12.6, 34.7 or 70.1 mg/kg bw per day for females, respectively), or 14 days (actual doses of 4.5, 13.5, 34.5 or 69.5 mg/kg bw per day for males and 4.5, 12.6, 34.7 or 70.0 mg/kg bw per day for females, respectively). All animals were observed daily for mortality and clinical or behavioural signs, and weekly for a detailed clinical examination, body weight, food consumption and ophthalmic examinations. Blood collection was carried out on day 7 (interim sacrifice group) and day 14 (terminal sacrifice group) to assess haematology and clinical chemistry. All the animals were euthanized, and gross pathological examinations and organ weight measurements were performed. Histopathology evaluations were limited to the tissues from the high dose and control group animals, and tissues with gross findings from all animals. Due to the presence of lesions in the adrenals, jejunum and mesenteric lymph nodes in the high-dose animals, these organs were evaluated at the low, mid and intermediate high-dose groups. No mortality or systemic clinical signs of toxicity were evident in any of the animals. At all dose levels, body weights, body weight changes and food consumption remained unaffected by treatment.

There were no treatment-related changes in haematology and coagulation parameters at the tested dose levels for the interim and terminal sacrifice groups. A treatment-related increase in total bilirubin levels was observed in the 80 mg/kg bw per day males and females euthanized on day 8, and in the 40 and 80 mg/kg bw per day females euthanized on day 15. A significant reduction was observed in the triglyceride levels in the 40 and 80 mg/kg bw per day males and females euthanized on days 8 and 15. A similar effect on triglyceride levels was also noted in the 15 mg/kg bw per day male rats euthanized on day 15. A treatment-related decrease in total cholesterol levels was noted in the 80 mg/kg bw per day

males and in the 40 and 80 mg/kg bw per day females at both intervals (days 8 and 15). A treatment-related increase in liver weights was noted in the 40 and 80 mg/kg bw per day females euthanized on day 8, and in both males and females euthanized on day 15 at the 40 and 80 mg/kg bw per day dose levels. The terminally euthanized females from the 15 mg/kg bw per day group also showed an increase in liver weights when compared with the control group (11–14% greater than the control). An increase in adrenal weights (absolute and relative) was noted in the 40 and 80 mg/kg bw per day treated females at both the interim and terminal sacrifice. No treatment-related gross pathological lesions were observed in any of the treated groups at the interim or terminal sacrifice. Treatment-related lymphangiectasis (lacteal dilatation) was noted in the jejunum in the 80 mg/kg bw per day dose males and females at the terminal sacrifice. Lymphangiectasis of mesenteric lymph nodes was observed in the 80 mg/kg bw per day females at the terminal sacrifice. In the adrenals, there was a dose-dependent minimal to moderate reduction in corticular fine vacuolation in the 40 and 80 mg/kg bw per day females at both the interim and terminal sacrifice. A no-observed-adverse-effect level (NOAEL) of 5 mg/kg bw per day (actual dose: 4.50 mg/kg bw per day for males and 4.56 mg/kg bw per day for females) was identified, based on reduction of triglyceride levels in males and increase in liver weight in females treated with 15 mg/kg bw per day on day 15 *(7)*.

In a GLP-compliant study, selamectin (99.6% purity) was administered for 28 days in the diet to Sprague Dawley rats (4/sex per dose). Males were administered selamectin at doses of 0, 15, 40 or 80 mg/kg bw per day (actual dose of 0, 14.6, 41.2 or 75.9 mg/kg bw per day), and females received selamectin at doses of 0, 5, 15 or 40 mg/kg bw per day (actual dose of 0, 4.5, 15.0 or 40.9 mg/bw per day). General health, mortality and moribundity were checked twice daily; cage-side clinical observations were made once daily; and observations for detailed clinical signs, body weights, body weight changes and food consumption were performed weekly. Functional observational battery (FOB) examinations were performed in an abbreviated assessment on day 2 postdose, and full assessments were made on pretreatment and week 4 postdose. Clinical pathology parameters (haematology, coagulation, clinical chemistry and urinalysis) were collected on day 28 or 29.

No test article-related mortality or clinical signs were noted during the study. There were no test article-related changes in mean body weight, mean body weight gain or food consumption for males during the study. However, there was a treatment-related decrease in absolute mean body weights on day 28 for all groups of treated females compared with controls. In addition, there was a decrease in mean body weight gain for females, which reached a level of statistical significance at 40 mg/kg bw per day for the total study period compared with controls. During the study, there were no correlating changes in mean food

consumption for females; no statistically significant or toxicologically relevant differences in FOB parameters in treated groups compared with controls; and no test article-related changes in haematology parameters. For clinical chemistry, there were slight but statistically significant increases in alkaline phosphatase (ALP) and total bilirubin for 40 and 80 mg/kg bw per day males, in ALP for 15 and 40 mg/kg bw per day females, and in total bilirubin for 40 mg/kg bw per day females compared with controls. However, there were no histological correlations and all values were within historical control ranges. In addition, there were statistically significant decreases in cholesterol and triglycerides for 40 mg/kg bw per day females, which were slightly below the normalised historical control ranges. There were no test article-related changes in urinalysis parameters. Oral administration of selamectin resulted in no test article-related gross pathology or microscopic changes in the standard histopathology tissues or in the evaluated nervous system tissues. Relative adrenal gland weights were higher in females at 40 mg/kg bw per day but lacked a microscopic correlation. Based on these results, a NOAEL could not be identified for males. A NOAEL of 15 mg/kg bw per day was identified for females, based on reduction of mean body weight and mean body weight gain, and increases in ALP and total bilirubin, and an increase of relative adrenal gland weights at 40 mg/kg bw per day *(8)*.

A GLP-compliant 13-week combined neurotoxicity/toxicity study was conducted using Sprague Dawley rats (10/sex per dose), given selamectin (purity 97.9%) in the diet for 13 weeks. Males received selamectin at doses of 0, 15, 40 or 80 mg/kg bw per day (actual doses of 0, 14.6, 39.1 or 75.4 mg/kg bw per day), and females received selamectin at doses of 0, 1, 15 or 40 (actual doses of 0, 0.8, 15.9 or 39.4 mg/kg bw per day). General health, mortality and moribundity were checked twice daily; cage-side clinical observations were made once daily; and observations for detailed clinical signs were made weekly. Body weights, body weight changes and food consumption assessments were performed at least once weekly. Ophthalmic examinations were performed on day –3 and during the last week of the treatment period (day 91). Clinical pathology parameters (haematology, coagulation, clinical chemistry and urinalysis) were collected on day 92. At termination, necropsy, organ weight and histopathologic examinations were performed.

There was no mortality, and no selamectin-related clinical signs were noted during the study. In addition, there were no selamectin-related changes in mean body weight, mean body weight gain or food consumption during the study. No selamectin-related changes were noted during ophthalmic examinations, and no changes in haematology or urinalysis parameters were noted during the study. There were statistically significant increases in ALP for the 40 and 80 mg/kg bw per day males and the 15 and 40 mg/kg bw per day females compared with controls. There was a trend for an increase in total bilirubin for treated males compared

with controls. However, all values were within historical control ranges. There were statistically significant decreases in cholesterol for 80 mg/kg bw per day males and 40 mg/kg bw per day females, and in triglycerides for 40 and 80 mg/kg bw per day males and 40 mg/kg bw per day females, which were slightly below the normalised historical control ranges. There were notable gross pathology findings, organ weight changes and/or histopathology findings at 40 mg/kg bw per day or more in males and at 15 mg/kg bw per day or more in females. The following test article-related changes in pathology were seen: at 15 mg/kg bw per day or more, higher adrenal gland weights (females), and higher liver weights, lymphatic dilatation and hepatocellular vacuolation (females); at 40 mg/kg bw per day or more, pale discolouration in the liver, higher liver weights, hepatocellular vacuolation (Oil-Red-O positive) (males), lymphatic dilatation in the small intestine (males), erythrocytosis in the mesenteric lymph nodes (males) and adrenal cortical hypertrophy (females); at 80 mg/kg bw per day, higher adrenal gland weights (females), lymphatic dilatation in the mesenteric lymph node and adrenal cortical hypertrophy (males).

A NOAEL was identified at 15 mg/kg bw per day (actual dose of 14.6 mg/kg bw per day) for males, based on the increase of ALP and decrease of cholesterol and triglycerides, and at 1 mg/kg bw per day (actual dose of 0.8 mg/kg bw per day) for females, based on the increase in ALP, and decrease of cholesterol and triglycerides, and alterations in liver and lymphatic dilatation in the small intestine, and adrenal cortical hypertrophy *(10)*.

In a GLP-compliant 3-month toxicity study, Sprague Dawley rats (5/sex per dose) were administered selamectin (86.6% purity) in sesame oil by oral gavage at doses of 0, 5, 15 or 80 mg/kg bw per day (males), and 0, 5, 15 or 40 mg/kg bw per day (females). Animals were observed daily for clinical signs of toxicity, and body weights and food consumption were recorded weekly. Ophthalmic examinations were performed pre-dose and at week 13 postdose. Clinical pathology parameters (haematology, serum chemistry and urinalysis) were performed during weeks 7 and 13. At the end of the dosing period, all surviving rats were euthanized and necropsied. Organ weights were recorded and histopathologic examinations were performed for selected organs.

Two high-dose males (80 mg/kg bw per day) and one intermediate-dose male (15 mg/kg bw per day) died during the study, but the author of the study report suggested that this was unrelated to the treatment. Sporadic salivation occurred in all treatment groups, including controls. However, excessive incidences of salivation were seen in the higher dose males and females. Selamectin had no effect on body weight, food consumption, ophthalmic examinations or urinalysis. Slight increases (1.3–1.4 times higher than control) in mean white blood cell counts and mean lymphocyte counts occurred in the high-dose males on days 42 and 86, and in the high-dose females on day 86. Mean

neutrophil counts increased (1.5–1.6 times higher than control) in the high-dose males on days 42 and 86. In addition, a slight increase (1.1–1.2 times higher than control) in mean fibrinogen concentrations was observed in the high-dose males on days 42 and 86, and in the high-dose females on day 86. Slight increases in mean serum alanine aminotransferase (ALT), ALP and total bilirubin were observed for high-dose males and females. Additionally, in the intermediate-dose group a slight increase (1.2–1.6 times higher than control) in ALT (females) and ALP (males and females) was observed. Mean total protein and globulin (GLOB) values were slightly decreased (0.8 times higher than control) in the high-dose males on day 86. This decrease in GLOB caused a slight increase (1.1 times higher than control) in the albumin to globulin (A/G) ratio. The A/G ratio was increased (1.1 times higher than control) in the high-dose females (40 mg/kg bw per day) on day 42, due to slightly increased (not statistically significant) albumin and slightly decreased (not statistically significant) GLOB concentration in these females compared with controls. Mean serum cholesterol levels were decreased (0.4 times higher than control) in the high-dose females on days 42 and 86. Mean serum triglyceride concentrations were decreased (0.3–0.8 times higher than control) in the low-dose females (5 mg/kg bw per day) on day 42, in the intermediate-dose females on days 42 and 86, and in the high-dose males (80 mg/kg bw per day) and females (40 mg/kg bw per day) on days 42 and 86. Mean glucose concentrations were slightly decreased (0.8–0.9 times higher than control) in the high-dose males on day 86, and in the high-dose females on days 42 and 86.

Mean absolute and relative liver and adrenal gland weights were increased (1.2–1.5 × control) in the 40 and 80 mg/kg bw per day males and females. Mean liver weights were also slightly increased (1.1 × control) in intermediate-dose females (15 mg/kg bw per day). Histologically, mild to moderate fatty change was observed in the liver of intermediate- and high-dose males (40 and 80 mg/kg bw per day, respectively) and high-dose females (40 mg/kg bw per day). The fatty liver change was correlated with an increase in mean absolute liver weight and hepatic ALT and ALP levels in the intermediate-dose females, and high-dose males (80 mg/kg bw per day) and females (40 mg/kg bw per day). Lymphatic dilation in the jejunum and duodenum was observed in males and females in the high- (80 and 40 mg/kg bw per day, respectively) and intermediate-dose group (15 mg/kg bw per day). In addition, adrenal gland hypertrophy with lipid-depleted, hypereosinophilic cortical cells was observed in the high-dose males and females. One intermediate-dose male (40 mg/kg bw per day) had adrenal cortical necrosis. The NOAEL in this study was 5 mg/kg bw per day, based on increased salivation; increases in ALT, ALP and total bilirubin levels; decreases in cholesterol and triglyceride levels; fatty liver changes with correlating increases

in mean absolute and relative liver weights and hepatic enzyme levels; and small intestine lymphangiectasia (lymphatic dilation) *(9)*.

(ii) **Dogs**

In a GLP-compliant 14-day oral range-finding toxicity study, male and female beagle dogs were administered selamectin (93.3% purity) in sesame oil, by oral gavage, at doses of 0 (1 male and 2 females), 10 (2 males and 1 female), 30 (1 male and 2 females) and 80 mg/kg bw per day (2 males and 1 female). Each day, all the dogs were observed for clinical signs, and their food intake was estimated. Their body weights were recorded on days 1, 4, 7, 10 and 13. Blood pressure, heart rate and electrocardiogram (ECG) recordings were performed before the study started and during the first week of treatment. Measurements were performed before and about 5 hours after treatment. Plasma drug concentrations were measured after the first and 13th doses. Haematology and plasma clinical chemistry investigations were performed before the start and at the end of the study. On day 15, the dogs were euthanized and necropsied, and several organs were weighed. A histopathological examination was carried out on a range of tissues. There was no mortality during the study (days 0–14). Diarrhoea and reduced food intake were observed in all treatment groups. Emesis or regurgitation was observed in the 30 and 80 mg/kg bw per day groups. There was evidence of emesis and diarrhoea before dosing. There was no treatment-related body weight loss in the 10 and 30 mg/kg bw per day groups; the animals of the 80 mg/kg bw per day group had body weight losses of 8% at the end of the study. There were no treatment-related effects observed in blood pressure, heart rate, ECG and haematology measurements. There were increases in ALP, ALT and aspartate amino transferase (AST) in one male dog in the 30 mg/kg bw per day and one female dog in the 80 mg/kg bw per day group. ALT values for the male dog and all values (ALP, ALT and AST) for the female dog were outside the limits of the laboratory historical range. Slightly higher relative liver weight than controls was observed in the 80 mg/kg bw per day group female, but this was within the 5–95% interval of the laboratory historical data. There were no treatment-related necropsy findings. Multiple foci of necrosis in the periportal areas of the liver were observed in the 80 mg/kg bw per day group female. A NOAEL of 30 mg/kg bw per day was identified in female dogs, based on increases in ALP, ALT and AST, with correlating necrosis in the periportal areas of the liver at 80 mg/kg bw per day *(30)*.

In a GLP-compliant study, selamectin (86.6% purity) in sesame oil was administered daily by oral gavage to beagle dogs (4/sex per dose) at doses of 0, 5, 15 and 40 mg/kg bw per day for 3 months. Before the start of the study, all animals were dosed with sesame oil only (1 mL/kg bw) for 7 consecutive days.

Dogs were observed daily for clinical signs and food consumption, and body weight was measured weekly. Ophthalmoscopic examinations were performed on pretreatment and on day 79 (males) and day 80 (females) postdose. Physical examinations, vital signs, ECG and blood pressure measurements were monitored once prior to the study and again on days 28 and 84. Haematology and serum chemistry parameters were monitored once prior to the study and again on days 31 and 87 postdose. Urinalysis parameters were also monitored once prior to the study and on days 29/30 and 85/86 postdose. At the conclusion of the dosing period, all dogs were euthanized, necropsied and examined for gross abnormalities. Selected organs (kidneys, liver, testes, adrenals, pituitary, ovaries, brain and heart) were weighed, and a comprehensive set of tissues was collected for microscopic examination.

All animals survived to study termination. Emesis, salivation and loose stools were the only noteworthy effects observed in the study. Emesis appeared to be treatment related (5/8 animals in each treatment group) but not dose related. Physical and ophthalmoscopic examinations revealed no adverse effects of the treatment. There were no treatment-related effects on body weight, food consumption, serum chemistry, haematology, coagulation, urinalysis parameters, vital signs, blood pressure or ECG tracings. There were no treatment-related findings at necropsy, and microscopic examination of tissues from all dose groups revealed no morphological changes associated with administration of selamectin. A NOAEL was identified at 5 mg/kg bw per day, based on salivation in the two higher dose groups *(31)*.

2.2.3 Long-term studies of toxicity and carcinogenicity

In a 1-year GLP-compliant study, selamectin (98.7% purity) was administered in the diet to Sprague Dawley rats (20/sex per dose) for up to 369 consecutive days. Males were given doses of 0, 1, 5, 15 or 40 mg/kg bw per day and females were given doses of 0, 0.3, 1, 5 or 15 mg/kg bw per day. Animals were observed daily for clinical signs, and weekly for occurrence of palpable masses. Body weights, body weight gains, food consumption and test article consumption were recorded weekly. Ophthalmic evaluations were performed for 2 weeks pretreatment and for all treated animals near the end of the study (week 51 for males and 50 for females). Clinical pathology parameters (haematology, serum chemistry and urinalysis) were performed during weeks 12, 25 and 52 (when coagulation evaluation was also performed); at the end of the dosing period, all surviving rats were weighed and then euthanized. Gross necropsy findings and organ weights were recorded, and histopathologic examinations and neuropathologic examinations were performed.

There was no mortality related to selamectin treatment; clinical observations or effects on palpable masses, food consumption, coagulation or urinalysis; and ophthalmic, neuropathologic or macroscopic findings. Lower body weight gains related to selamectin treatment were intermittently noted in the 40 mg/kg bw per day group males and in the 15 mg/kg bw per day group females throughout the study. The lower body weight gains resulted in body weights at the end of the dosing period that were 6.9% lower than the control group for the 40 mg/kg bw per day group males and 6.5% lower than the control group for the 15 mg/kg bw per day group females. Haematology alterations were noted in the 15 mg/kg bw per day group females during week 52; they consisted of a lower red blood cell count (–10.0%), haemoglobin value (–10.8%) and haematocrit value (–9.7%). Higher ALP values were noted in the 15 and 40 mg/kg bw per day males during weeks 12, 25 and 52; a dose–response was evident, and the magnitude of difference from the control group increased over time in the affected groups, resulting in ALP values that were 77.6% and 192.4% higher than the control group, respectively, during week 52. Lower cholesterol and triglyceride values (–30.1% and –53.1%, respectively) were noted in the 40 mg/kg bw per day group males during week 52, and lower triglyceride values were noted in the 5 and 15 mg/kg bw per day group females during week 25 (–39.4% and –46.5%, respectively) and week 52 (–22.1% and –36.0%, respectively).

Higher adrenal gland weights (absolute and relative to body and brain weight) were observed in the 40 mg/kg bw per day group males. Higher liver weights (absolute and relative to body and brain weight) were noted in the females at 5 mg/kg bw per day or more. Higher uterus/cervix weights (absolute and relative to body and brain weight) were noted in the 15 mg/kg bw per day treated females. No histologic or clinical chemistry correlations were identified for these organ weight changes. Treatment-related histology findings consisted of minimal and moderate lymphangiectasia in the jejunum of the 40 mg/kg bw per day group males. This finding was characterized by dilated lymphatics (lacteals) within and surrounded by the lamina propria of the villi and at the base of the villi. A NOAEL was identified at 1 mg/kg bw per day, based on lower cholesterol and triglyceride values and changes in haematology parameters in the 5 and 15 mg/kg bw per day in females and 40 mg/kg bw per day males; higher liver weights (absolute and relative to body and brain weight) were found in the females from groups treated with selamectin at 5 mg/kg bw per day or more, and higher uterus/cervix weights (absolute and relative to body and brain weight) in 15 mg/kg bw per day treated females *(11)*.

Carcinogenicity studies were not submitted by the sponsor, nor were they found in the scientific literature.

2.2.4 Genotoxicity

Five studies (four in vitro and one in vivo) were performed to examine the genotoxicity potential of selamectin. The genotoxicity of selamectin was tested in GLP-compliant assays, including an Ames test, an in vitro mammalian gene mutation test, an in vitro cytogenetic assay primary human lymphocyte and an in vivo micronucleus assay mouse. The results of all studies were negative. The results are summarized in Table 1.

2.2.5 Reproductive and developmental toxicity

(a) Reproductive toxicity

(i) Rats

In a GLP-compliant dosage range-finding study for the multigeneration reproductive toxicity study, selamectin (99.6% purity) was administered in the diet to pregnant Sprague Dawley rats (8 females/dose) at doses of 0, 5, 10, 25 or 50 mg/kg bw per day from presumed GD0 to postpartum day 21 (for rats that delivered a litter) or GD25 (for rats that did not deliver a litter). Day 0 of lactation was defined as the day of birth (i.e. PND0). F1 generation pups were directly exposed after they began consuming food (starting on ~PND14). All animals were observed twice daily for mortality and moribundity. Clinical observations, body weights and food consumption were recorded at appropriate intervals. Natural delivery observations (including litter size and viability), pup body weights, pup developmental landmarks, gross necropsy findings and organ weights were also recorded.

No deaths related to exposure to selamectin occurred in the parental (P) generation females. One female in the 10 mg/kg bw per day exposure group was euthanized (unscheduled) on day 1 of lactation because no pups survived. This early euthanasia of the animal was considered unrelated to the test substance because it was not dose-dependent. All other females survived until scheduled euthanasia. There were no treatment-related macroscopic observations in the females that survived to scheduled euthanasia at any dose. Adverse effects possibly related to selamectin treatment included effects in F0 dams. At 25 mg/kg bw per day, there was a slight increase in the absolute and relative mean liver weights. At 50 mg/kg bw per day, a significantly reduced body weight gain during gestation (23% below control) was verified; also, an increased number of females were found to have clinical signs of mild dehydration and hunched posture during lactation, and there was an increase in the absolute and relative mean liver weights. At 50 mg/kg bw per day, one litter had pups with microphthalmia and/or lenticular opacity; a pup from this litter was delivered without a tail. In conclusion, at 25 mg/kg bw per day, liver weights were slightly increased, and at 50 mg/kg bw per day, findings included adverse clinical signs, reduced body

Table 1
Results of genotoxicity assays on selamectin

Study type, species, GLP status	Concentration range or dose	Purity	Treatment regimen	Results	Reference
In vitro assays					
Microbial reverse mutation (Ames) assay *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98 and TA 100 GLP-compliant	Preliminary test: 1–5 mg/plate Definitive test: 0.002 to 1 mg/plate	Not specified	Minimal incubation of 60 hours, with and without metabolic activation (S9)	Negative with and without S9	Guzzie (1994) *(32)*
Microbial reverse mutation (Ames) assay *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98 and TA 100, and *Escherichia coli* strain WP2 uvrA GLP and OECD 471 compliant	Preliminary test: 1.5, 5.0, 15, 50, 150, 500, 1500 and 5000 µg/plate Definitive test: 15, 50, 150, 500, 1500, 5000	99.6%	48-hour to 72-hour incubation, with and without metabolic activation (S9)	Negative with and without S9	Dakoulas (2016)
Mammalian cell gene mutation assay CHO cells GLP-compliant	Preliminary test: 4–3500[a] µg/mL –S9 4–3500[b] µg/mL +S9 Definitive test: 3–10 µg/mL –S9 3–30 µg/mL +S9	Not specified	Preliminary test: 5-hour treatment, then cells were washed, trypsinized and counted; 106 cells from each culture were plated and incubated until day 2 and then washed, trypsinized and counted Definitive test: same as above with a count on day 2 or 3; cells were then subcultured every 2–3 days and mutant selection was performed on day 7	Negative with and without S9	Guzzie (1995) *(34)*
Cytogenetic assay primary human lymphocytes GLP-compliant	Preliminary test: 3.9–500 µg/mL –S9 3.9–500 µg/mL +S9 Definitive test: 3–25[c] µg/mL –S9 4–30[d] µg/mL +S9	Not specified	Preliminary test: 24-hour incubation 3-hour incubation Definitive test: 24-hour incubation 3-hour incubation	Negative with and without S9	Guzzie (1995) *(35)*
In vivo assay					
Micronucleus assay mouse GLP-compliant	_	Not specified	0 (vehicle), 250, 500, 1000, 2000[e] mg/kg per day for 3 days, orally by gavage	Negative	Guzzie (1995) *(36)*

CHO: Chinese hamster ovary; GLP: good laboratory practice; OECD: Organisation for Economic Co-operation and Development.
[a] Concentrations ≥85 µg/mL had incomplete compound solubility in culture medium, excessive toxicity and were not evaluated.
[b] Concentrations ≥145 µg/mL had incomplete compound solubility in culture medium, excessive toxicity and were not evaluated.
[c] Concentrations ≥8 µg/mL were not evaluated due to insufficient numbers of mitotic cells for analysis.
[d] Concentrations ≥19 µg/mL were not evaluated due to insufficient numbers of mitotic cells for analysis.
[e] Due to excessive deaths in the 2000 mg/kg bw per day dose group, micronuclei induction was evaluated in the three lowest dose groups.

weight gains and increased liver weight in dams. A NOAEL of 10 mg/kg bw per day was identified, based on an increase in liver weights in dams. These findings

were used in the selection of doses for a definitive two-generation reproductive toxicity study of selamectin in rats *(14)*.

In a GLP-compliant multigeneration toxicity study, selamectin (99.6% purity) was administered in the diet to groups of male and female Sprague Dawley rats (25/sex per group) at doses of 0, 5, 15 or 50 mg/kg bw per day. The actual consumed doses of selamectin in F0 and F1 males and females (at pre-mating, gestation and lactation) were close to targeted doses. Dosing began 70 days prior to mating. Selamectin was administered to offspring selected to become the F1 parental generation following weaning (i.e. on PND21). The F0 and F1 males continued to receive the test and control diets throughout mating, until the day of euthanasia. The F0 and F1 females continued to receive the control and test diets throughout mating, gestation and lactation, until the day of euthanasia. For both generations (F1 and F2), eight pups per litter (4 per sex, when possible) were selected on PND4 to reduce the variability among the litters. Offspring (25/sex per group) from the pairing of the F0 animals were selected prior to PND21, to constitute the F1 generation. F0 males and females were exposed for 131–134 consecutive days, and F1 males and females were exposed for 133–148 consecutive days. Estrus cyclicity was determined by vaginal lavage prior to cohabitation. All F0 and F1 females were allowed to deliver and rear their pups, until weaning on PND21. All animals were observed twice daily for mortality and moribundity. Clinical observations, body weights and food consumption were recorded at appropriate intervals. F1 and F2 pups were sexed at birth. Developmental landmarks (balanopreputial separation and vaginal patency) and neurobehavioural evaluations (FOB) were evaluated on PND60 for the F1 rats selected to constitute the F1 generation. Non-selected F1 pups and all F2 pups were euthanized and necropsied on PND21, and selected organs were weighed for one pup per sex per litter for each generation. Surviving F0 and F1 parents underwent complete detailed gross necropsies, and selected organs were weighed. Spermatogenic end-points (sperm motility, morphology and numbers) were recorded for all F0 and F1 males; also, ovarian primordial follicle counts were recorded for all F1 females in the control and high-dose groups, and for all F1 females suspected of reduced fertility. Designated tissues from all F0 and F1 parents in the control and high-dose groups were examined microscopically.

At 50 mg/kg bw per day, two F0 females and one F1 female died or were euthanized due to dystocia. One F1 male in the 50 mg/kg bw per day group died during the mating period. At necropsy, the animal was found to have a swollen spleen, but the cause of death could not be determined. In the 15 mg/kg bw per day group, two F1 males were euthanized in extremis and one F1 female was found dead. One of the two males, euthanized during the post-mating period, had a benign pituitary tumour; the other male and the female died on PND28, early in the post-weaning period. None of the deaths in the 15 mg/kg bw per

day group was considered to be treatment-related. No F0 animals in the 15 mg/kg bw per day group were found dead or euthanized in extremis. At all levels of exposure, no selamectin-related clinical findings were observed in the F0 and F1 generations; no selamectin-related effects were observed in the F0 and F1 male and female mating and fertility, male copulation or female conception indices, mean estrus cycle lengths, mean pre-coital intervals, spermatogenesis parameters or mean gestation lengths; and no selamectin-related effects were observed on F1 maturational (balanopreputial separation, vaginal patency or FOB) parameters. No selamectin-related macroscopic findings were noted in the F0 generation, or in F1 or F2 pups that were found dead or were euthanized for the scheduled necropsy on PND21. Non-adverse minimal to mild lymphatic dilation of the intestines was seen at low incidences in all selamectin-treated F0 and F1 groups.

In relation to the F0 generation, effects possibly related to selamectin exposure were observed at the dose of 50 mg/kg bw per day: one female died prior to euthanasia and one female was euthanized due to dystocia. Lower mean body weights and body weight gains (males) and gestational body weights and body weight gains (females) were observed. Other changes in the 50 mg/kg bw per day group included higher mean liver weights in males and females, but without corresponding microscopic changes; higher mean adrenal gland weights in males and females, with corresponding hypertrophy in the adrenal cortex in females only; and higher mean numbers of unaccounted-for implantation sites (i.e. increase in post-implantation loss).

Considering the F1 generation, at the dose of 50 mg/kg bw per day one female died due to dystocia and one male was found dead with a swollen spleen. The administration of 50 mg/kg bw per day of selamectin was also associated with lower mean body weights and body weight gains in both females and males (but because of the magnitude of the changes, the effects on mean body weight gains in the males were not considered adverse), and gestation body weights and body weight gains (females). The dose of 50 mg/kg bw per day of selamectin was also associated with higher mean liver weights in males (including two with swollen livers) and females, and corresponding microscopic changes (hepatocellular vacuolation). Also associated with the higher dose of selamectin were higher mean adrenal gland weights in females, with corresponding non-adverse hypertrophy in the adrenal cortex and higher mean numbers of unaccounted-for implantation sites (i.e. a presumptive increase in post-implantation loss). The administration of 15 mg/kg bw per day of selamectin was associated with higher mean liver weights in males and females; however, no corresponding microscopic changes were detected.

Concerning the F1 and F2 offspring, effects possibly related to selamectin treatment at 15 mg/kg bw per day were the lower mean spleen weights in F1 females, and lower mean thymus weights in F2 males and females. Treatment

with 50 mg/kg bw per day was correlated with lower live litter sizes for F1 and F2 pups (related to a presumptive increase in post-implantation loss), lower mean postnatal survival through weaning in F1 (87.2% versus 94.8% in controls) and F2 litters (90.8% versus 99.5% in controls), lower mean birth weights (F1 pups) and/or body weight gains (F1 and F2 pups), and lower mean thymus gland and spleen weights in F1 and F2 pups.

The NOAEL for female reproductive toxicity was 15 mg/kg bw per day, based on the findings of dystocia resulting in mortality or euthanasia, and a higher rate of presumptive post-implantation loss resulting in a lower mean number of pups born at 50 mg/kg per day. The NOAEL for male reproductive toxicity was 50 mg/kg bw per day, the highest dose tested. Based on lower mean body weight gains at 50 mg/kg bw per day, the NOAEL for parental toxicity was considered to be 15 mg/kg bw per day. The NOAEL for neonatal and developmental toxicity was considered to be 5 mg/kg bw per day, based on lower mean pup survival, mean pup body weights and/or body weight gains and lower mean pup spleen and thymus weights at 15 mg/kg bw per day *(15)*.

In a dietary developmental neurotoxicity GLP-compliant study, selamectin (97.9%) was administered in the diet to groups of pregnant Sprague Dawley rats (25/group) at doses of 0, 5, 15 or 50 mg/kg bw per day from GD6 through postpartum day 21 (for rats that delivered a litter) or GD25 (for rats that did not deliver a litter). Day 0 of lactation was defined as the day of birth. F1 generation pups were directly exposed after they began consuming food (starting on about PND14). All animals were observed twice daily for mortality and moribundity. Clinical observations, body weights and food consumption were recorded at appropriate intervals; gross necropsy findings were recorded at termination.

In the F1 animals, no treatment-related mortalities or clinical findings were observed. Further, there were no selamectin-related effects on body weight, body weight gain, food consumption values or necropsy observations in the F1 animals at any exposure level. Terminal body weights and brain weights, the ratio of the brain weight to the terminal body weight, neuromorphometric parameters and neurohistopathology in F1 rats on postpartum Days 21 and 71 (±2 days) were not affected by maternal exposure to selamectin up to 50 mg/kg bw per day. One and two F0 females from the 5 and 50 mg/kg bw per day groups, respectively, died during the lactation period. The deaths in the 50 mg/kg bw per day group were considered to be selamectin-related because they were accompanied by clinical signs of toxicity, whereas the death in the 5 mg/kg bw per day group was considered to be incidental. A rat in the control group was euthanized during the gestation period because of adverse clinical observations. No F0 animals in the 15 mg/kg bw per day group were found dead or euthanized in extremis. No maternal necropsy findings or pup necropsy alterations were observed during

the pre-weaning period. In relation to the F0 generation, effects possibly related to selamectin exposure were observed at the dose of 50 mg/kg bw per day: two females died prior to euthanasia and exhibited clinical signs of toxicity (i.e. dehydration, hunched posture and piloerection). In addition, lower mean body weight gains during gestation and lactation, and reduced body weights during lactation were observed. Considering the F1 generation, the administration of 50 mg/kg bw per day of selamectin was associated with an increased number of stillborn pups (resulting in a reduced number of liveborn pups), and an increased number of pups found dead or presumed cannibalized on PND0. In addition, a decreased viability index at PND4 was observed. Decreased mean pup body weights on PND4, 6 and 9 was also detected.

The NOAEL for maternal toxicity was 15 mg/kg bw per day, based on mortalities, decreased body weight gains and an increase in the number of dams with stillborn pups at 50 mg/kg bw per day. The NOAEL for neonatal and developmental toxicity was considered to be 15 mg/kg bw per day, based on a decreased viability index in the 50 mg/kg bw per day group at PND4, and reduced pup weights on PND4, 6 and 9 *(16)*.

In a GLP-compliant modified segment I fertility and reproduction study, selamectin (purity not specified) in sesame oil was administered to Sprague Dawley rats (20/sex per group) by oral gavage at doses of 0, 10, 25 or 60 mg/kg bw per day, from 4 or 2 weeks prior to mating for the F0 males and females, respectively. Selamectin administration continued through mating, gestation and lactation for the F0 females. F0 males were dosed for 106–109 days, and euthanized on days 107–110. The F1 generation were dosed directly by oral gavage, beginning on PND21. All F0 dams were allowed to litter. The F1 pups were weighed on PND1, 4, 7, 10, 14 and 21. Body weights were recorded every 3–4 days through 27 days of age, and weekly thereafter. Food consumption was recorded weekly beginning on day 28. Postnatal development was assessed for all F1 pups for the appearance of developmental indices and reflex behaviours. Also conducted were a FOB evaluation and tests of motor activity, learning and memory. FOB parameters were evaluated on PND22, 23 or 24, and an assessment of motor activity was performed on PND27 or 28. The F1 animals were also assessed for reproductive success. Beginning on PND21, two pups per sex per litter were retained, and received a daily dose of selamectin equal to the dose given to their F0 dam. The F1 males were dosed for a total of 83–96 days, and F1 females for a total of 83–100 days. The F2 pups were evaluated for body weight and viability through to PND10.

Reduced fertility in the high-dose group

The higher dose of selamectin was toxic in F0 females, affecting gestational body weight gain, fertility, litter size and food consumption during lactation. Selamectin did not affect the ability of the animals (male and females) to mate, and there were no effects on length and number of estrus cycles, sperm parameters, copulation or the mean length of cohabitation. Fertility was reduced in the 60 mg/kg bw per day group, suggesting that this effect is not male mediated. Gestation length increased in the 25 and 60 mg/kg bw per day groups, and in the 60 mg/kg bw per day group there were decreases in the number of pups born and the number alive on PND1 and 4. Significant decreases in mean pup weights were noted for both male and female offspring in the 60 mg/kg bw per day group, whereas offspring of the animals treated with 10 and 24 mg/kg bw per day had an increase in mean body weight. In relation to developmental parameters, the day of achievement of surface righting was earlier in the pups in the 60 mg/kg bw per day group. There was a dose-related trend for the earlier opening of the vagina, eye opening and incisor eruption, which reached significance in the 25 mg/kg bw per day group. FOB parameters were not affected between the treatment groups and the control.

Considering the adult F1 generation, the mean food consumption was higher when compared with the controls in both the 10 and 25 mg/kg bw per day groups. There were no treatment-related effects on the estrus cycle, duration of cohabitation, copulation or fertility rate. Fertility was comparable among the treatment groups and the controls. Gestation was significantly prolonged in the 25 mg/kg bw per day group. No other reproductive parameters were affected. No treatment mortalities were noted, with the exception of one female that died of dystocia in the 25 mg/kg bw per day group.

The NOAEL for female reproductive toxicity was 10 mg/kg bw per day, based on prolonged gestational length and fetal toxicity in the 25 and 60 mg/kg bw per day groups, and decreased postnatal survival at 60 mg/kg bw per day. The NOAEL for male reproductive toxicity was 60 mg/kg bw per day, the highest dose tested. The NOAEL for neonatal and developmental toxicity was considered to be 10 mg/kg bw per day, based on earlier opening of the vagina, eye opening and incisor eruption *(37)*.

(ii) **Dogs**

In a GLP-compliant study, male beagle dogs (10/group) were administered topically with either 18 mg/kg bw of the commercial formulation of selamectin (Revolution, 93.3/92.4% purity) or placebo (0.9% saline). Selamectin was applied to the dogs every 14 days for at least seven treatments prior to mating; this continued every 14 days during mating (each male was mated with two untreated females). Semen sample was collected during the treatment and mating period.

Each male received a total of 17 treatments. The treated males were assessed for clinical and general health observations (including mating behaviour), body weights, physical examination, clinical pathology and semen quality during the treatment and mating period. Litters resulting from the mating of treated males with untreated females were evaluated within 24 hours of birth for congenital abnormalities, litter size and pup viability. Numerical data resulting from the semen and litter assessments were statistically evaluated. Group means and standard deviations were calculated for all other quantitative data.

There were no deaths of treated adult males during the study, and no treatment-related changes in any of the variables observed or measured. Health observations, body weights, physical examinations, clinical pathology, semen quality and litter assessments were comparable between the selamectin and placebo groups. Thus, topical administration of selamectin to breeding adult male beagle dogs did not cause negative health or reproductive effects *(38)*.

In a GLP-compliant study, female beagle dogs (22/group) were administered topically either 18 mg/kg bw of the commercial formulation of selamectin (Revolution, 93.3/92.4% purity) or placebo (0.9% saline), once every 28 days. Following the administration of at least two treatments, 20 female dogs from each group were mated with untreated males. Following mating, two regimens were followed (10 dogs/compound per regimen), with treatments administered either on post-mating days 1, 29, 57, 85 and 113, or on post-mating days 15, 43, 71, 99 and 127. The selamectin-treated female dogs were assessed for clinical and general health observations (including mating behaviour), body weight, physical examination and clinical pathology. Litters resulting from the mating of treated females with untreated males were evaluated for congenital abnormalities, litter size and puppy viability on the day of parturition and at the end of the study (when puppies were 42 days old). Puppies remained with their mothers until 42 days old (weaning), during which time they were assessed for general health, body weight gain and physical health. Conception rate, whelping index and weaning index were calculated. The total number of puppies and number of live puppies per treatment group were evaluated.

There were no deaths of the treated adult females during the study, and no treatment-related changes in any of the variables observed or measured. Health observations, body weights, physical examination, clinical pathology, conception rate and weaning index were comparable between the selamectin and placebo groups. More total puppies per litter and more live puppies per litter were born to selamectin-treated females assigned to the regimen in which post-mating treatments began on day 15. Thus, topical administration of selamectin to breeding adult female beagle dogs did not cause negative health or reproductive effects *(39)*.

(b) **Developmental toxicity**

(i) Rats

In a non-GLP-compliant dose range-finding prenatal developmental toxicity study, selamectin (99.6% purity) was administered in CMC and Tween 80, by oral gavage, from GD6 to GD19 to groups of eight female Sprague Dawley rats at doses of 0, 20, 32, 50 or 80 mg/kg bw per day. All animals were observed twice daily for mortality and moribundity. Clinical observations, body weights and food consumption were recorded at appropriate intervals. On GD20, a laparohysterectomy was performed on each female. The uteri, placentae and ovaries were examined, and the number of fetuses, early and late resorptions, total implantations and corpora lutea were recorded. Gravid uterine weights were recorded, and net body weights and net body weight changes were calculated. The fetuses were weighed, sexed and examined for external malformations and developmental variations.

Mean body weight losses and lower mean body weights, body weight gains, net body weight, net body weight gain, gravid uterine weight, and food consumption were noted in the 80 mg/kg bw per day group. No differences in these parameters were detected in animals treated with 20, 32 or 50 mg/kg bw per day of selamectin. Increased incidences of enlarged adrenal glands, dark red discolouration of the adrenal glands, and dark red contents in the uterus and/or vagina were noted in the 80 mg/kg bw per day group, and were correlated to higher post-implantation loss in this group. No remarkable macroscopic findings were noted in the 20, 32 and 50 mg/kg bw per day groups. A higher mean litter proportion of post-implantation loss, resulting in fewer viable fetuses, was noted in the 80 mg/kg bw per day group. Mean fetal body weights (by sex and combined) in this group were also 16.2% lower than the control group. Intrauterine growth and survival in the 20, 32 and 50 mg/kg bw per day groups were similar to the control group. External malformations were noted in two fetuses in the 50 mg/kg bw per day group (exencephaly with open eyelids and mandibular micrognathia with microstomia) and in one fetus in the 80 mg/kg bw per day group (a malrotated hindlimb). The NOAEL for maternal toxicity was 50 mg/kg bw per day, based on lower mean body weights and body weight gains, reduced food consumption, enlargement of adrenal glands, dark red discolouration of the adrenal glands, and dark red contents in the uterus and/or vagina observed in the 80 mg/kg bw per day group. The NOAEL for embryo/fetal toxicity was 32 mg/kg bw per day based on teratogenicity noted in two fetuses in the 50 mg/kg bw per day group *(13)*.

In a GLP-compliant developmental toxicity study, selamectin (99.6% purity) was administered in CMC and Tween 80, by oral gavage, from G6 to GD19 to groups of 25 pregnant female Sprague Dawley rats at doses of 0, 6.67, 20 or 60 mg/kg bw per day. All animals were observed twice daily for mortality and

moribundity. Clinical observations, body weights and food consumption were recorded at appropriate intervals. On GD20, a laparohysterectomy was performed on each surviving female. The uteri, placentae and ovaries were examined, and the number of fetuses, early and late resorptions, total implantations and corpora lutea were recorded. The fetuses were weighed, sexed and examined for external malformations and developmental variations. For each litter, one half of the fetuses were examined for visceral abnormalities, and the other for skeletal abnormalities.

No effects of selamectin treatment were observed on clinical signs, macroscopic findings, intrauterine growth or survival at any dose level. Increased incidences of red material around the nose at 20 and 60 mg/kg bw per day were not considered adverse, given the absence of any other signs of toxicity. A selamectin-related lower mean maternal body weight gain was noted in the 60 mg/kg bw per day group during GD12–15, with correspondingly lower mean food consumption during GD9–15. In addition, a lower mean net body weight gain was noted in this group compared with the control group. The decrease in body weight gain and the lower mean net body weight gain were considered to be non-adverse, given the absence of effect on absolute mean body weight. No treatment-related macroscopic findings were noted at any dosage level. Intrauterine growth and survival at 6.67, 20 and 60 mg/kg bw per day were not affected by administration of selamectin. At 60 mg/kg bw per day, there was an increase in the skeletal developmental variations of sternebra(e) nos. 5 and/or 6 unossified, reduced ossification of the vertebral arches and reduced ossification of the skull. The NOAEL was considered to be 20 mg/kg per day, based on the reduced maternal body weight gain in dams from the 60 mg/kg bw group. The NOAEL for embryo/fetal toxicity was 20 mg/kg bw per day, based on the increase in the skeletal developmental variations of sternebra unossified, reduced ossification of the vertebral arches and reduced ossification of the skull in the 60 mg/kg bw per day group *(40)*.

In a GLP-compliant maternal toxicity study, groups of 12 pregnant female Sprague Dawley rats were dosed with selamectin (purity not specified) in sesame oil once daily, by oral gavage, at levels of 0, 10, 40 or 60 mg/kg bw per day. Body weight and food consumption were recorded daily. Seven animals per group were dosed from GD6 to GD17 and underwent caesarean sections on GD21. For each dam, the numbers of corpora lutea, implantation sites, resorption and live and dead fetuses were recorded. Viable fetuses were weighed individually and examined for external anomalies. The remaining five rats per group were dosed from GD6 through PND8–10.

Selamectin at 60 mg/kg bw per day resulted in decreased food consumption, increased incidence of resorptions, decreased body weights in the dams allowed to deliver, and dystocia in one dam. There was no effect on

mean fetal weights, and no treatment-related external anomalies were noted. One dam in each of the 10 and 60 mg/kg bw per day groups neglected its litter and was euthanized. Maternal body weights decreased from GD16 through PND10 in the 60 mg/kg bw per day group. Females treated with 60 mg/kg bw per day of selamectin showed decreased litter sizes and increased numbers of stillborn pups. The NOAEL for maternal toxicity was 40 mg/kg bw per day, based on decreased food consumption, decreased body weights and increased incidence of resorptions in females treated with 60 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 40 mg/kg bw per day, based on decreased litter size and increased numbers of stillborn pups in the 60 mg/kg bw per day group *(12)*.

In a GLP-compliant teratology study, groups of 20 pregnant Sprague Dawley rats were dosed with selamectin (purity not specified) in sesame oil once daily, by oral gavage, at doses of 0, 10, 40 or 60 mg/kg bw per day. The animals were observed at least twice daily for clinical signs. Body weights and food consumption were recorded daily. Caesarean sections were performed at GD21. Each uterus was weighed prior to the removal of the fetuses. Fetuses and placentas were weighed and examined for external anomalies. For each litter, half of the pups were examined for visceral anomalies, and half for skeletal anomalies and degree of skeletal ossification.

Maternal survival was unaffected by selamectin administration, but at 60 mg/kg bw per day, decreases in maternal food consumption and gestational body weight gain, and increases in resorption and post-implantation loss indicated some maternal toxicity. There were no effects on corpora lutea, implantation sites, number of viable fetuses, litter size, fetal body weight or sex ratio. There was an increase in mean placental weight in the 40 and 60 mg/kg bw per day groups. There were no effects of selamectin administration on the skeletal system of the fetuses, and no effects on soft tissue abnormalities except for the heart, where an increased incidence of enlarged right atria and fibrin material in the thoracic cavity occurred in some fetuses in the 40 and 60 mg/kg bw per day groups, and occasional other cardiac defects occurred in the 60 mg/kg bw per day group. The NOAEL for maternal toxicity was 40 mg/kg bw per day, based on a decrease in food consumption, gestational body weight gain, and increases in resorption and post-implantation loss at 60 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 10 mg/kg bw per day, based on increased placental weight, increased incidence of enlarged right atria, and the occurrence of fibrin material in the thoracic cavity in fetuses at doses of 40 and 60 mg/kg bw per day, plus occasional other cardiac defects that occurred at 60 mg/kg bw per day *(41)*.

(ii) Rabbits

In a non-GLP-compliant oral dose range-finding developmental toxicity study, New Zealand rabbits (6 females/group) were administered selamectin (99.6% purity) in 1.0% CMC and 0.5% Tween 80, by oral gavage, in doses of 0, 20, 32, 50 or 80 mg/kg bw per day during GD7–28. All animals were observed twice daily for mortality and moribundity. Clinical observations, body weights and food consumption were recorded at appropriate intervals. A laparohysterectomy was performed on each female, and blood samples were collected from one viable fetus per litter. The uteri, placentae and ovaries were examined, and the numbers of fetuses, early and late resorptions, total implantations and corpora lutea were recorded. Gravid uterine weights were recorded, and net body weights and net body weight changes were calculated. The fetuses were weighed and examined for external malformations and developmental variations.

All animals in the 0, 20, 32, 50 and 80 mg/kg bw per day groups survived to the scheduled euthanasia and necropsy on GD29. Mean maternal body weights, body weight gains, food consumption, net body weights, net body weight gains and gravid uterine weights in the 20, 32, 50 and 80 mg/kg per day groups were similar to those in the control group. At the scheduled necropsy on GD29, no remarkable internal findings were observed at dosage levels of 20, 32, 50 and 80 mg/kg bw per day. Intrauterine growth and survival, and external fetal morphology, were unaffected by administration of selamectin at 20, 32, 50 and 80 mg/kg bw per day. The NOAEL was 80 mg/kg bw per day, the highest dose tested, because there was a lack of adverse maternal or developmental toxicity in New Zealand white rabbits *(17)*.

In a non-GLP-compliant oral dose range-finding developmental toxicity study, New Zealand rabbits (3 females/group) were administered selamectin (99.6% purity) in 1.0% CMC and 0.5% Tween 80, by oral gavage, in doses of 0, 125, 250, 500 and 1000 mg/kg bw per day during GD7–28. All animals were observed twice daily for mortality and moribundity. Clinical observations, body weights and food consumption were recorded at appropriate intervals. On GD29, a laparohysterectomy was performed on each surviving female. The uteri, placentae and ovaries were examined, and the number of fetuses, early and late resorptions, total implantations and corpora lutea were recorded. The fetuses were weighed and examined for external malformations and developmental variations. Additionally, blood samples for toxicokinetic evaluation were taken from each female on GD7, 14, 21, 27 and 29, and from one viable fetus per litter at the time of laparohysterectomy.

No effects of selamectin treatment were observed on macroscopic findings at necropsy, or in intrauterine growth and survival. In the 125 mg/kg bw per day group, one female was euthanized in extremis on GD12, following one day of severe body weight loss with corresponding negligible food consumption,

decreased defecation, laboured respiration and rales; however, necropsy findings suggested that this moribundity was due to an intubation error. Adverse effects possibly related to selamectin treatment were abortion in one female in each group treated with 250, 500 and 1000 mg/kg bw per day; animals from the 500 and 1000 mg/kg bw per day groups showed dose-related body weight losses in all females during GD7–10; body weight losses were also observed in two of three females at 125 mg/kg bw per day on these same days. Regarding the fetuses from females treated with 500 and 1000 mg/kg bw per day, two fetuses had omphalocele. A NOAEL could not be identified due to maternal or embryo/fetal toxic effects (or both) observed in all dose groups *(18)*.

In a GLP-compliant prenatal developmental toxicity study, New Zealand rabbits (24–25 females/group) were administered selamectin (99.6% purity) in 1.0% CMC and 0.5% Tween 80, by oral gavage, in doses of 0, 20, 60 or 180 mg/kg bw per day during GD7–28. All animals were observed twice daily for mortality and moribundity. Clinical observations, body weights and food consumption were recorded at appropriate intervals. On GD29, a laparohysterectomy was performed on each surviving female. The uteri, placentae and ovaries were examined, and the number of fetuses, early and late resorptions, total implantations and corpora lutea were recorded. Gravid uterine weights were recorded, and net body weights and net body weight changes were calculated. The fetuses were weighed, sexed and examined for external, visceral and skeletal malformations and developmental variations.

No effects of selamectin treatment were observed on macroscopic findings at necropsy, or in intrauterine growth and survival, or fetal morphology. No treatment-related effects on maternal body weights, body weight gains, gravid uterine weights or food consumption were noted in the 20 and 60 mg/kg bw per day groups. At 180 mg/kg bw per day, one non-gravid female was euthanized in extremis on GD24 following body weight loss (15.4%) with corresponding reduced food consumption and decreased defecation; because of the absence of other signs of adverse maternal toxicity at this dose level, the moribund condition of this female was not attributed to treatment. At 60 mg/kg bw per day, one female aborted on GD19; because this occurred in a single animal in a manner that was not dose-related, it was not considered to be related to selamectin. Another female at 60 mg/kg bw per day was removed from the study on GD8 owing to body weight loss and reduced food consumption prior to the initiation of dosing. Selamectin-related mean body weight loss following the initiation of treatment (GD7–10 interval) was noted in the 180 mg/kg bw per day group compared with the control group, resulting in a lower mean body weight gain when the entire treatment period (GD7–28) was evaluated. In addition, a treatment-related greater mean net body weight loss was noted in the 180 mg/kg bw per day group compared with the control group. Mean food consumption in the 180 mg/kg bw

per day group was lower than the control group throughout the treatment period. Gravid uterine weight was similar to the control group at 180 mg/kg bw per day. No treatment-related effects on maternal body weights, body weight gains, net body weights, net body weight gains, gravid uterine weights or food consumption were noted in the 20 and 60 mg/kg bw per day groups. The NOAEL for maternal toxicity was 60 mg/kg bw per day based on reduced food intake and body weight at 180 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 180 mg/kg bw per day, based on the absence of effects observed in all dose groups. No evidence of teratogenicity was observed *(19)*.

2.2.6 Special studies

(a) Neurotoxicity

Three neurotoxicology studies are described: two of these studies were subchronic and chronic neurotoxicity studies, which were conducted in conjunction with standard toxicity tests of the same duration. The standard toxicity and neurotoxicity studies were conducted on separate animals. The toxicokinetic and main toxicity studies and results are described in other sections of this monograph (Sections 2.1.1 and 2.2, respectively).

In a GLP-compliant study, selamectin (97.9% purity) was administered for 13 weeks in the diet to Sprague Dawley rats (4/sex per dose). Males received doses of 0, 15, 40 or 80 mg/kg bw per day (actual doses of 0, 14.6, 39.1 or 75.4 mg/kg bw per day), and females received doses of 0, 1, 15 or 40 (actual dose of 0, 0.8, 15.9 or 39.4 mg/kg bw per day). Full FOB assessments were performed on all animals assigned to the neurotoxicity study phase; the assessments were performed at about the same time of day, on the 8th day of life and during weeks 2, 4, 8 and 13 (days –9, 13, 26, 53 and 88 for males and days –7, 15, 27, 55 and 90 for females). These assessments included observation of individual rats in the home cage, while being removed from the home cage and in a standard arena for open field observations, and functional tests for sensory, neuromuscular and physiological changes. The motor activity assessment included an assessment of potential effects on the pattern of habituation within each 60-minute session. At termination, all rats were perfused in situ with fixative, and multiple sections of the brain, spinal cord and peripheral nerves were examined. A variety of stains were used including hematoxylin and eosin, Bielschowsky stain (a silver stain for demonstrating selected neuronal processes, including axons) and immunohistochemistry for glial fibrillary acidic protein for astrocytes within brain and spinal cord sections. No selamectin-related changes were observed in any of the neurobehavioural tests or histopathology evaluations of the nervous system. Thus, the NOAEL for neurotoxicity was 80 mg/kg bw per day for males and 40 mg/kg bw per day for females *(10)*.

In a 1-year GLP-compliant study, selamectin (98.7% purity) was administered in the diet to Sprague Dawley rats (20/sex per control and 10/sex per dose group). Males received selamectin at doses of 0, 1, 5 or 40 mg/kg bw per day and females at doses of 0, 0.3, 5 or 15 mg/kg bw per day. FOB, motor activity and neuropathology evaluations were conducted in a similar manner to that described for the 13-week neurotoxicity study *(10)*. Microscopic evaluations were limited to animals in the control and high-dose groups. The FOB and motor activity assessments were conducted prior to initiation of exposure, and after about 1, 3, 6, 9 and 12 months of exposure. For this neurotoxicity component of the study, the author found no selamectin-related effects on clinical observations, FOB, motor activity and neuropathologic findings at any of the dose levels tested. Selamectin-related lower body weight gains were intermittently noted in the 40 mg/kg bw per day toxicity and neurotoxicity group males, and the 15 mg/kg bw per day females. For the neurotoxicology component of this study, lower body weight gains were 6.7% and 4.6% lower than the control group for the 40 mg/kg bw per day males and 15 mg/kg bw per day females, respectively. These minimal effects on body weights were not considered to be adverse. Based on the lack of adverse findings, the NOAEL for neurotoxicity following chronic exposures was 40 mg/kg bw per day for males and 15 mg/kg bw per day for females, the highest doses tested. This NOAEL is identical to that of the toxicology component of this chronic study *(11)*.

In a GLP-compliant dietary developmental neurotoxicity study, selamectin (97.9% purity) was administered in the diet to groups of pregnant female Sprague Dawley rats (25/group) at doses of 0, 5, 15 or 50 mg/kg bw per day from GD6 through postpartum day 21 (for rats that delivered a litter) or GD25 (for rats that did not deliver a litter). F1 generation pups were directly exposed after they began consuming food (starting on ~PND14). In addition to standard developmental toxicology end-points, the F1 rats were evaluated for neurobehavioural and neuropathological findings. The neurobehavioural end-points were conducted on 10 or 20 rats per sex per dose group; they included detailed clinical observations, learning and memory (passive avoidance at weaning and Morris water maze in adults), behavioural ontogeny (motor activity 3 days prior to weaning), acoustic startle reactivity and habituation. At weaning and terminal euthanasia, 10 F1 rats per sex per dose group were perfused in situ for neuromorphometric (all dose levels) and neurohistopathological (control and high dose) evaluation. Microscopic evaluations of sections of spinal cord and the peripheral nervous system were restricted to adult animals. Two F0 females from the 50 mg/kg bw per day group died during the lactation period. The deaths were considered to be treatment-related, because they were accompanied by clinical signs of toxicity (dehydration, hunched posture and piloerection). Other possible adverse effects observed in the F0 animals were reduced body weight and body

weight gain in the 50 mg/kg bw per day group. In the F1 generation, possible adverse effects were increased number of stillborn pups and pups found dead, decreased viability index and decreased pup body weights in the 50 mg/kg bw per day group. The NOAEL for maternal toxicity was considered to be 15 mg/kg bw per day, based on these findings. The NOAEL for neonatal and developmental toxicity was considered to be 15 mg/kg bw per day, based on the decreased viability index in the 50 mg/kg bw per day F1 group at PND4 and reduced pup weights. The NOAEL for developmental neurotoxicity was considered to be 50 mg/kg bw per day, the highest dose tested, because there were no selamectin-related effects on any behavioural or neuropathology end-points *(16)*.

(b) **Mechanistic studies**

(i) In vitro pharmacology of selamectin on the human gamma aminobutyric acid A channel

In a non-GLP-compliant study, selamectin (purity not specified) and ivermectin (purity not specified) were assessed for functional activity (agonist and antagonist) at a recombinantly expressed human gamma aminobutyric acid (GABA) channel subtype (α1β2γ2). Using automated voltage-clamp electrophysiology, selamectin produced non-significant inward currents at the GABACl (1.2 ±0.2% of maximal GABA) while also failing to block GABA-induced currents. In contrast, ivermectin produced statistically significant inward currents (2.5 ±0.2% of maximal GABA and 3.6 ±0.8% of maximal GABA AUC) that were sustained without apparent desensitization of the GABACl. Ivermectin was shown to potently block GABACls, thus demonstrating distinct functional properties when compared with selamectin. These data demonstrated distinct pharmacological profiles for selamectin and ivermectin, despite belonging to the same chemical class *(42)*.

(ii) Rat liver transcriptomic analysis

Liver samples were collected from the rat dietary repeat dose toxicity studies for gene expression and transcriptomic benchmark dose (BMD) analysis. BMD analysis was conducted using BMDExpress software *(43)*. Liver samples were obtained from the toxicokinetic cohorts (3–6/sex per group) from the 2-week study *(7)*, 28-day study *(8)*, 90-day study *(10)* and 1-year chronic study *(11)*.

Considering the non-GLP-compliant 2-week study (doses of 0, 5, 15, 40 or 80 mg/kg bw per day, for both male and female) and 28-day dietary study (doses of 0, 15, 40 or 80 mg/kg bw per day for males, and 0, 5, 15 or 40 mg/kg bw per day for females), a number of conclusions were obtained from assessment of the transcriptional response. The genomic findings were consistent with the histopathology and morphology findings, in that no discrete pathways of toxicity were evident in the differentially expressed gene profiles. The specific changes that

were observed were limited to the female samples at either time point. In males, changes in gene expression appeared to be completely nonspecific with regard to functional ontology cellular processes. In females, changes in gene expression were primarily restricted to changes in genes associated with cholesterol and lipid metabolism and were limited solely to genes that were induced (i.e. upregulated) in expression relative to controls. All reduced expression (i.e. downregulated genes) was associated with a broad range of processes related to cell cycle pathways, cell signalling pathways, apoptosis and some immune system responses. All of these apparent genomic changes would be consistent with the slight decrease in body weight in females (related to generalized cell stress responses), and changes in cholesterol and triglyceride levels. But there was no genomic evidence of a strong or discrete toxic response. Additionally, several changes in cytochrome P450 genes were noted; these appeared to be largely sex specific differences in expression, regardless of exposure period. This may indicate fundamental differences in metabolism of selamectin that could be directly related to the differences observed in response to exposure between the two sexes *(44)*.

Considering the non-GLP-compliant 90-day dietary study (selamectin at doses of 15, 40 or 80 mg/kg bw per day in males, and 1, 15, and 40 mg/kg bw per day in females), a number of conclusions were obtained from the transcriptional response assessment, which presented the results of differential gene expression and BMD analyses. Female rats showed little differential gene expression after 90 days of selamectin treatment at any dose, and there was no significant functional ontology enrichment using female rat differentially expressed genes. Males showed little differential gene expression at the lowest dose, but several hundred or more differentially expressed genes at the 40 and 80 mg/kg bw per day doses. In males, differentially expressed genes from 80 mg/kg bw per day exposure indicated ontology enrichment in the metabolic process with upregulated genes, and cell locomotion and adhesion with downregulated genes. The gene-based BMD response in males and females was consistent with that seen at earlier time points (i.e. at 14 days and 28 days), with only minor shifts in overall responsiveness to selamectin treatment. In both sexes, at 90 days, BMD results were shifted on average towards a somewhat lower response. BMD pathway enrichment again indicated similar, although slightly more sensitive, responses in the integrated software suite (MetaCore) pathway maps ontology categories, but overall trends were similar to those seen at 28 days in both sexes *(44)*.

Considering a non-GLP-compliant evaluation of a 1-year study (selamectin at doses of 0, 1, 5, 15 or 40 mg/kg bw per day in males and 0, 0.03, 1.5 or 15 mg/kg bw per day in females), a number of conclusions were obtained from the transcriptional response and benchmark dose analyses. For females, the differential gene expression (DGE) at 15 mg/kg bw per day was maximal overall in the 1-year exposures, relative to previous 14, 28 and 90-day exposures,

but was largely nonspecific in terms of cellular functional response. For males, DGE was less than the maximum observed at 80 mg/kg bw per day and 90 days exposure, but did show ontology enrichment for metabolic processes similar to those enriched with female DGEs at 14 and 28 days. The observed differential expression and ontology results were consistent with the previous findings (14, 28 and 90-day analyses), in showing no evidence of discrete pathways of toxicity evident in the differentially expressed gene profiles. Benchmark dose analyses indicated a lowest summary point of departure (POD) was that derived from gene base summaries (20 genes with lowest BMD in each sex of 0.19 mg/kg bw per day in females, and 0.56 mg/kg bw per day in males).

(iii) Investigational pathology

Two non-GLP-compliant investigative pathology studies were performed to verify the pathogenesis for intestinal lymphangiectasia related to selamectin administration. In both studies, female Sprague Dawley rats were dosed by oral gavage or in the diet, with selamectin (96.3% purity), in high doses (80 or 100 mg/kg bw per day), in different schedules of administration and euthanasia. However, data from both studies could not be assessed, because one study had technical problems, and the other had a low incidence and severity of lymphangiectasia of the duodenum, jejunum and ileum, and occurrence of acute toxicity *(45, 46)*.

2.3 Microbiological effects

Selamectin is a family of macrolides known for their anthelmintic activities and traditionally believed to be inactive against all bacteria *(47)*. A few studies reported that some members of the family – ivermectin, selamectin and moxidectin – are bactericidal against mycobacterial species, including multidrug-resistant and extensively drug resistant clinical strains of *Mycobacterium tuberculosis (48, 49)*). However, no data are available on the antimicrobial impact of selamectin on the human gut microbiome. Hence, it was not possible to derive a microbiological acceptable daily intake (ADI).

2.4 Observations in humans

No studies with selamectin have been conducted in humans. No information on accidental or intentional poisoning in humans is available.

3. Comments

3.1 Biochemical data

The pharmacokinetics of selamectin was studied in rats, rabbits and dogs. Most of the investigations were done as part of the toxicological studies. There was no information on the metabolism and distribution of selamectin when administered by oral route in laboratory animals.

In two radiolabel studies in dogs *(23, 24)*, using a single topical dose, selamectin was eliminated in the faeces (18–20% of the dose) and urine (1–3% of the dose); most of the radioactivity in the faeces was the parent compound (39% females and 64% males). Unchanged selamectin accounted for the radioactivity observed in urine. Several oxidative O-desmethyl metabolites of selamectin were detected in faeces, together constituting less than 10% of the total radioactivity present. In an in vitro comparative metabolism study in liver microsomes of male and female rats, female rabbits, dogs, salmon, and male and female humans, the metabolites produced were variously derived from pathways involved in mono-oxidation on different positions, *O*-demethylation and/or epoxidation accompanying epoxide ring hydroxylation. No unique metabolites were produced by salmon or human samples.

The oral administration in male and female rats of a single 6 mg/kg bw dose of a solution of selamectin revealed similar mean C_{max} values of 1190 ng/mL and 1260 ng/mL, respectively. The $AUC_{0-\infty}$ values, however, were different, being 18 600 ng/hour per mL in males and 46 200 ng/hour per mL in females. The plasma half-lives also differed between males and females but were similar to the respective half-lives following intravenous dosing at 7.6 hours and 22.0 hours. The oral bioavailability was 58.5% in males and 43.7% in females *(4)*. These pharmacokinetic differences between male and female rats were apparent in both short-term and long-term studies (including a multigeneration study), so that females had substantially greater systemic exposure to selamectin than males *(7-11)*. The plasma half-lives given above *(4)* may not be the terminal half-lives, given that plasma levels in the 1-year repeated-dose oral toxicity study in rats reached a steady state only after 274–365 days *(11)*. In contrast to rats, no sex differences in pharmacokinetics were apparent in dogs *(20)*.

In a maternal toxicity study in rats, the analysis of milk and maternal plasma samples indicated that selamectin concentrations were similar in both *(12)*. Exposure to selamectin in utero was evaluated in rats; fetal plasma concentrations were 20–35% of those seen in the dams *(16)*. In rabbits, fetal plasma concentrations of selamectin were similar to plasma concentrations in dams *(18)*.

3.2 Toxicological data

In rats and mice, the oral LD_{50} of selamectin was greater than 1600 mg/kg bw *(26)*.

Changes in lipid metabolism and increases in weights and lesions of the liver and adrenal gland were demonstrated in both sexes of rats and dogs after short-term or long-term oral exposure to selamectin. In rats, lymphangiectasia (a dilatation of the lymphatic vessels) occurred in the small intestine; however, the pathogenesis of this process could not be determined.

In a 14-day non-GLP-compliant dose-ranging study, selamectin was administered in the diet to rats at nominal doses of 0, 5, 15, 40 or 80 mg/kg bw per day during 14 days (actual doses of 4.5, 13.5, 34.5 or 69.5 mg/kg bw per day for males and 4.5, 12.6, 34.7 and 70.0 mg/kg bw per day for females, respectively). A NOAEL of 4.5 mg/kg bw per day was identified, based on reduction of triglyceride levels in males and increase in liver weight in females at higher doses *(7)*.

In a 28-day study, selamectin was administered in the diet to rats. Males received selamectin at nominal doses of 0, 15, 40 or 80 mg/kg bw per day (actual doses of 0, 14.6, 41.2 or 75.9 mg/kg bw per day), and females received selamectin at nominal doses of 0, 5, 15 and 40 mg/kg bw per day (actual doses of 0, 4.5, 15.0 and 40.9 mg/kg bw per day). A NOAEL of 14.6 mg/kg bw per day was identified, based on reduction of mean body weight and mean body weight gain, increases in alkaline phosphatase and total bilirubin, and increase of relative adrenal gland weights at higher doses *(8)*.

In a 13-week study in rats, animals received diets containing selamectin at nominal doses in males of 0, 15, 40 or 80 mg/kg bw per day (actual doses of 0, 14.6, 39.1 or 75.4 mg/kg bw per day), and in females of 0, 1, 15 or 40 mg/kg bw per day (actual doses of 0, 0.8, 15.9 or 39.4 mg/kg bw per day). The NOAEL was 0.8 mg/kg bw per day, based on increase in ALP, and decrease in cholesterol and triglycerides, alterations in liver, lymphangiectasia in the small intestine and adrenal cortical hypertrophy in females at higher doses *(10)*.

In a 3-month study of toxicity, rats were administrated selamectin orally by gavage at doses of 0, 5, 15 or 80 mg/kg bw per day (male), and at doses of 0, 5, 15 or 40 mg/kg bw per day (female). The NOAEL was 5 mg/kg bw per day, based on increases in alanine transaminase, alkaline phosphatase and total bilirubin levels, decreases in cholesterol and triglyceride levels, and the occurrence of fatty liver and lymphangiectasia of the small intestine at 15 mg/kg bw per day *(9)*.

In a 3-month study of toxicity in dogs, animals received selamectin by oral gavage at doses of 0, 5, 15 and 40 mg/kg bw per day. The NOAEL was 5 mg/kg bw per day, based on consistent salivation observed at higher doses. This effect started to occur after 6 days of dosing *(31)*.

In a 1-year study of toxicity and neurotoxicity, rats were fed diets containing selamectin to achieve doses of 0, 1, 5, 15 or 40 mg/kg bw per day (male) and 0, 0.3, 1, 5 or 15 mg/kg bw per day (females). The NOAEL was 1 mg/kg bw per day, based on lower cholesterol and triglyceride values, alterations in haematology parameters, and higher liver and uterus/cervix weights in females treated with 5 mg/kg bw per day. There was no evidence of neurotoxicity *(11)*.

No specific 2-year toxicity studies or carcinogenicity studies were provided.

The genotoxicity of selamectin was investigated in an adequate range of assays, both in vivo *(36)* and in vitro *(32-35)*. No evidence of genotoxicity was found.

The Committee concluded that selamectin is unlikely to be genotoxic.

In view of the lack of genotoxicity, any carcinogenicity would be secondary to prolonged preneoplastic damage – for which there was no evidence in a chronic (1-year) study in rats – and because other avermectins (e.g. abamectin) are not carcinogenic, the Committee concluded that selamectin is unlikely to pose a carcinogenic risk to humans from residues in the diet *(14)*.

In a two-generation reproductive toxicity study, rats received diets containing selamectin to achieve doses of 0, 5, 15 or 50 mg/kg bw per day. The NOAEL for reproductive toxicity was 50 mg/kg bw per day, the highest dose tested. The NOAEL for parental toxicity was 15 mg/kg bw per day, based on lower mean body weight gain at 50 mg/kg bw per day. The NOAEL for offspring toxicity was 5 mg/kg bw per day, based on lower mean pup survival, mean pup body weights and/or body weight gains, and pup lower mean spleen and thymus weights at 15 mg/kg bw per day *(15)*.

In a study of developmental neurotoxicity, selamectin was administered in the diet to groups of pregnant rats (25/group) at doses of 0, 5, 15 or 50 mg/kg bw per day from GD6 through PND21. Following delivery, first filial generation (F_1) pups were directly exposed after they began consuming food (starting on about PND14). The NOAEL for maternal toxicity was 15 mg/kg bw per day, based on mortalities, decreased body weight gains and an increase in the number of dams with stillborn pups at 50 mg/kg bw per day. The NOAEL for neonatal and developmental toxicity was 15 mg/kg bw per day, based on decreased viability index at PND4 and reduced pup weights on PND4, 6 and 9 in the 50 mg/kg bw per day group. The NOAEL for developmental neurotoxicity was 50 mg/kg bw per day, the highest dose tested *(16)*.

In a one-generation study of reproductive toxicity, selamectin was administered to rats by oral gavage at doses of 0, 10, 25 or 60 mg/kg bw per day, from 4 or 2 weeks prior to mating for the parental generation (F_0) males and females, respectively. Selamectin administration continued through mating, gestation and lactation for the F_1 females. F_0 males were dosed through study

days 106–109 and euthanized on days 107–110. All F_0 dams were allowed to litter. The NOAEL for female reproductive toxicity was 10 mg/kg bw per day, based on prolonged gestational length and fetal toxicity at 25 mg/kg bw per day. The NOAEL for male reproductive toxicity was 60 mg/kg bw per day, the highest dose tested. The NOAEL for offspring toxicity was 10 mg/kg bw per day, based on earlier vaginal opening, eye opening and incisor eruption at 25 mg/kg bw per day *(37)*.

In a dose range-finding non-GLP-compliant study of developmental toxicity, rats were administered selamectin via oral gavage at doses of 0, 20, 32, 50 or 80 mg/kg bw per day from GD6 to GD19. External malformations were noted in two fetuses in the 50 mg/kg bw per day group (exencephaly with open eyelids and mandibular micrognathia with microstomia) and one fetus in the 80 mg/kg bw per day group (a malrotated hindlimb). The NOAEL for maternal toxicity was 50 mg/kg bw per day, based on lower mean body weights and body weight gains, reduced food consumption, enlargement of adrenal glands, dark red discolouration of the adrenal glands, and dark red contents in the uterus and/or vagina, observed at 80 mg/kg bw per day. The NOAEL for teratogenicity was 32 mg/kg bw per day based on external malformations noted in two fetuses in the 50 mg/kg bw per day group *(13)*.

In a study of developmental toxicity, rats were administered selamectin via oral gavage at doses of 0, 6.67, 20 or 60 mg/kg bw per day from GD6 to GD19. The NOAEL was 20 mg/kg bw per day, based on the reduced maternal body weight gain in dams from the 60 mg/kg bw per day group. No malformations were observed in any dose group. The NOAEL for embryo/fetal toxicity was 20 mg/kg bw per day, based on increased incidences of skeletal variations (sternebra unossified, reduced ossification of the vertebral arches and reduced ossification of the skull) in the 60 mg/kg bw per day group *(40)*.

In a developmental toxicity study, pregnant rats were dosed with selamectin by oral gavage at levels of 0, 10, 40 or 60 mg/kg bw per day from GD6 to GD17. The NOAEL for maternal toxicity was 40 mg/kg bw per day, based on a decrease of food consumption and gestational body weight gain, and increases in resorption and post-implantation loss at 60 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 10 mg/kg bw per day based on reduced placental weight, and increased incidence of enlarged right atria and fibrin material in the thoracic cavity, which occurred in fetuses at 40 mg/kg bw per day. The NOAEL for teratogenicity was 40 mg/kg bw per day, based on an increased incidence of several cardiac malformations in fetuses at 60 mg/kg bw per day *(12)*. This may be a suitable basis for an acute reference dose (ARfD).

In a prenatal study of developmental toxicity, pregnant rabbits were administered selamectin by oral gavage at doses of 0, 20, 60 or 180 mg/kg bw per day from GD7 to GD28. The NOAEL for maternal toxicity was 60 mg/

Table 2
Summary of toxicity studies – selamectin

Species/study type (route of administration)	Doses (mg/kg bw per day)	Critical end-point	NOAEL (mg/kg bw per day)	LOAEL (mg/kg bw per day)
Rat				
13-week neurotoxicity/ toxicity study (dietary)	Male: 0, 15, 40 and 80 (actual doses: 0, 14.6, 39.1 and 75.4) Female: 0, 1, 15 and 40 (actual doses: 0, 0.8, 15.9 and 39.4)	Increase in adrenal and liver weights, increased activity of liver enzymes, decrease in cholesterol and triglyceride levels, hepatocellular vacuolation, adrenal hypertrophy, lymphangiectasia in the small intestine	1.0	15
3-month toxicity study (gavage)	Male: 0, 5, 15 and 80 Female: 0, 5, 15 and 40	Increase in adrenal and liver weights, dilatation in jejunum and duodenum, lymphangiectasia, increased activity of liver enzymes and bilirubin, decrease in cholesterol and triglycerides	5.0	15
1-year toxicity study (dietary)	Male: 0, 1, 5 and 40 Female: 0, 0.3, 5 and 15	Decrease in cholesterol and triglycerides, higher liver and uterus weights	1.0*	5.0
Two-generation reproductive toxicity study (dietary)	0, 5, 15 and 50	Reproductive toxicity: nil	50[a]	–
		Parental toxicity: lower mean body weight gain, higher liver weight	15	15
		Offspring toxicity: reduced pup weights, lower spleen and thymus weights	5.0	15
Developmental toxicity study (gavage)	0, 6.67, 20 and 60	Maternal toxicity: reduced body weight gain	20	60
		Developmental toxicity: increase in the skeletal developmental variations of sternebra unossified, reduced ossification of the vertebral arches and reduced ossification of the skull	20	60
Developmental toxicity study	0, 10, 40 and 60	Maternal toxicity: decrease of food consumption, decrease of body weight gain. Increases in resorption and post-implantation loss	40	60
		Developmental toxicity: increase in enlarged right atria and fibrin material in the thoracic cavity	10	40
		Teratogenicity: cardiac malformations	40**	60
Rabbit				
Developmental toxicity study (gavage)	0, 20, 60 and 180	Maternal toxicity: reduced body weight gain	60	180
		Developmental toxicity: nil	180[a]	–
Dog				
3-month toxicity study	0, 5, 15 and 40	Salivation	5.0	15

ADI: acceptable daily intake; ARfD: acute reference dose; bw: body weight; LOAEL: lowest-observed-adverse-effect level; NOAEL: no-observed-adverse-effect level.
* Pivotal study for the derivation of the ADI *(11)*.
** Pivotal study for the derivation of the ARfD *(37)*.
[a] Highest dose tested.

kg bw per day, based on body weight loss at 180 mg/kg bw per day within the first week of treatment. The NOAEL for embryo/fetal toxicity was 180 mg/kg bw per day, based on the absence of observed effects in all dose groups *(19)*.

The Committee concluded that selamectin is teratogenic at high dosages in rats but not in rabbits.

Studies relevant to the risk assessment are summarized in Table 2.

3.3 Microbiological data

No data for antimicrobial impact of selamectin on the human gut microbiome are available. However, considering the chemical structure and mode of action of the avermectin class, the Committee did not anticipate any adverse effects of selamectin residues on human gastrointestinal microbiota.

4. Evaluation

In the absence of a 2-year study or a carcinogenicity study, and lack of information about the effects of selamectin on adrenal glands (hypertrophy) and the pathogenesis of the lymphangiectasia in the small intestine, the Committee was unable to establish an ADI for selamectin. The Committee could not assure itself that there would be an adequate margin of safety from the use of selamectin as a veterinary drug.

The Committee established an ARfD of 0.05 mg/kg bw on the basis of a NOAEL of 5 mg/kg bw for salivation in a 3-month toxicity study in dogs, applying a safety factor of 100.

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ANNEX 1

Reports and other documents resulting from previous meetings of the Joint FAO/WHO Expert Committee on Food Additives

1. General principles governing the use of food additives (First report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 15, 1957; WHO Technical Report Series, No. 129, 1957 (out of print).
2. Procedures for the testing of intentional food additives to establish their safety for use (Second report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 17, 1958; WHO Technical Report Series, No. 144, 1958 (out of print).
3. Specifications for identity and purity of food additives (antimicrobial preservatives and antioxidants) (Third report of the Joint FAO/WHO Expert Committee on Food Additives). These specifications were subsequently revised and published as Specifications for identity and purity of food additives, Vol. I. Antimicrobial preservatives and antioxidants, Rome, Food and Agriculture Organization of the United Nations, 1962 (out of print).
4. Specifications for identity and purity of food additives (food colours) (Fourth report of the Joint FAO/WHO Expert Committee on Food Additives). These specifications were subsequently revised and published as Specifications for identity and purity of food additives, Vol. II. Food colours, Rome, Food and Agriculture Organization of the United Nations, 1963 (out of print).
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13. Specifications for the identity and purity of food additives and their toxicological evaluation: some emulsifiers and stabilizers and certain other substances (Tenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 43, 1967; WHO Technical Report Series, No. 373, 1967.

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ANNEX 2

Abbreviations used in the monographs

95/95 UTL	95/95 upper tolerance limit; upper limit of the one-sided 95% confidence interval over the 95th percentile of residue concentrations
ABRET	absolute number of reticulocytes
ADI	acceptable daily intake
AGAL	Australian Government Analytical Laboratories
ALP	alkaline phosphatase
ALT	alanine aminotransferase
ARfD	acute reference dose
AST	aminotransferase
AUC	area under the concentration–time curve
$AUC_{0-\infty}$	area under the concentration–time curve from time 0 extrapolated to infinite time
AUC_{0-100}	area under the concentration–time curve from time 0 to time of limit of quantification
BMD	benchmark dose
bw	body weight
CAC	Codex Alimentarius Commission (Codex)
CAS	Chemical Abstracts Service
CCPR	Codex Committee on Pesticide Residues
CCRVDF	Codex Committee on Residues of Veterinary Drugs in Foods
CIFOCOss	FAO/WHO Chronic Individual Food Consumption Database – Summary statistics
5-CL	5-chloro-8-hydroxyquinoline or 5-chloroquinolin-8-ol (CHQ)
7-CL	7-chloro-8-hydroxyquinoline
5-CLG	5-chloro-8-hydroxyquinoline glucuronide conjugate
5-CLS	5-chloro-8-hydroxyquinoline sulfate conjugate
C_{max}	maximum concentration
CMC	carboxymethylcellulose
CNS	central nervous system
5,7-DCL	5,7-dichloroquinolin-8-ol (DCHQ)
5,7-DCLG	5,7-dichloroquinolin-8-ol glucuronide conjugate
5,7-DCLS	5,7-dichloroquinolin-8-ol sulfate
DGE	differential gene expression
DNA	deoxyribonucleic acid
EC	emulsifiable concentrate

eq	equivalents
EU	European Union
F_0	parental generation
F_1	first filial generation
FAO	Food and Agriculture Organization of the United Nations
FOB	functional observational battery
FSCJ	Food Safety Commission of Japan
GABA	gamma aminobutyric acid
GABACl	gamma aminobutyric acid chloride channel
GD	gestational day
GEADE	global estimate of acute dietary exposure
GECDE	global estimate of chronic dietary exposure
GL	guideline
GLOB	total protein and globulin
GLP	good laboratory practice
GLuCl	glutamate-gated chloride channel
GVP	good practice in the use of veterinary drugs
HBGV	health-based guidance value
HPLC	high-performance liquid chromatography
HPLC-FL	high-performance liquid chromatography with fluorescence detection
HPLC-UV	high-performance liquid chromatography with ultraviolet detection
ICR	Institute of Cancer Research
IEDI	international estimate of daily intake
IP	identification point
IPCS	International Programme on Chemical Safety
ISO	International Organization for Standardization
IUPAC	International Union of Pure and Applied Chemistry
JECFA	Joint FAO/WHO Expert Committee on Food Additives
JMPR	Joint FAO/WHO Meeting on Pesticide Residues
LC-MS/MS	liquid chromatography coupled to tandem mass spectrometry
LD_{50}	median lethal dose
LOAEL	lowest-observed-adverse-effect level
LOD	limit of detection
LOQ	limit of quantification
m/z	mass-to-charge ratio
mADI	microbiological acceptable daily intake
mARfD	microbiological acute reference dose
MCHC	mean corpuscular haemoglobin concentration
MCV	mean corpuscular volume
MIC	minimum inhibitory concentration

MIC_{50}	minimum concentration required to inhibit the growth of 50% of organisms
MIC_{calc}	minimum inhibitory concentration derived from the lower 90% confidence limit for the mean MIC_{50} of the relevant genera for which the drug is active
MnRET	micronucleated reticulocyte
MOE	margin of exposure
MR	marker residue
MR:TR	marker residue to total residue
MR:TRR	marker residue to total radioactive residue
MRL	maximum residue limit
MS	mass spectrometry
NOAEC	no-observed-adverse-effect concentration
NOAEL	no-observed-adverse-effect level
NTP	National Toxicology Program
OECD	Organisation for Economic Co-operation and Development
PND	postnatal day
POD	point of departure
RER	rough endoplasmic reticulum
SD	standard deviation
SER	smooth endoplasmic reticulum
SPF	specific pathogen free
STMR	supervised trials median residues
T_{max}	time to reach the maximum concentration (C_{max})
TLC	thin layer chromatography
TR	total residue
TRR	total radioactive residue
TTC	threshold of toxicological concern
UDP	up-and-down procedure
UHPLC-HRMS	ultra-high-performance liquid chromatography coupled to a high-resolution mass spectrometer
UHPLC-MS/MS	ultra-high-performance liquid chromatography coupled to tandem mass spectrometry
USA	United States of America
UTL	upper tolerance limit
VICH	International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products
w/w	weight per weight
WHO	World Health Organization

ANNEX 3

Participants in the eighty-eighth meeting of the Joint FAO/WHO Expert Committee on Food Additives

World Health Organization (WHO) members

Professor (Emeritus) Alan R. Boobis, National Heart and Lung Institute, Imperial College London, London, United Kingdom of Great Britain and Northern Ireland (United Kingdom) (*Co-Chair*)

Dr Carl E. Cerniglia, Director, Division of Microbiology, National Center for Toxicological Research, Food and Drug Administration, Jefferson, United States of America

Mr G.J. (Johan) Schefferlie, Medicines Evaluation Board, Veterinary Medicinal Products Unit, Utrecht, The Netherlands (*WHO Rapporteur*)

Food and Agriculture Organization of the United Nations (FAO) members

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WHO experts

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Secretariat

Ms Gracia Brisco, Food Standards Officer, Joint FAO/WHO Food Standard Programme, Food and Agriculture Organization of the United Nations, Rome, Italy (*Codex Secretariat*)

Dr Hilary Cadman, Cadman Editing Services, Bellingen, Australia (*WHO technical editor*)

Dr Vittorio Fattori, Food Safety and Quality Unit, Agriculture and Consumer Protection Department, Food and Agriculture Organization of the United Nations (*FAO Secretariat*)

Dr Kevin Greenlees, Senior Advisor for Science and Policy, Center for Veterinary Medicine, US Food and Drug Administration, Maryland, United States of America (*Chair of Codex Committee on Residues of Veterinary Drugs in Foods*)

Dr Markus Lipp, Senior Food Safety Officer, Scientific Advice and Joint FAO/WHO Expert Committee on Food Additives (JECFA) Secretary, Agriculture and Consumer Protection Department, Food and Agriculture Organization of the United Nations, Rome, Italy (*FAO Secretariat*)

Mr Soren Madsen, Joint FAO/WHO Meeting on Pesticide Residues (JMPR) and JECFA Secretariat, Department of Food Safety and Zoonoses (FOS), World Health Organization, Switzerland (*WHO Secretariat*)

Annex 4

Recommendations on the substances on the agenda

Diflubenzuron (insecticide)

Acceptable daily intake	The Committee established an acceptable daily intake (ADI) of 0–0.02 mg/kg body weight (bw) – based on a no-observed-adverse-effect level (NOAEL) of 2 mg/kg bw per day for increased methaemoglobin and sulfhaemoglobin levels in a 2-year study of toxicity and carcinogenicity in rats; and increased methaemoglobin and sulfhaemoglobin levels, platelet counts and hepatic pigmentation in a 1-year study of toxicity in dogs – applying a safety factor of 100 (10 for interspecies variability and 10 for intraspecies variability).
Acute reference dose	The Committee reiterated the conclusion of the 81st meeting *(1)* that it was not necessary to establish an acute reference dose (ARfD), in view of the low acute oral toxicity and the absence of developmental toxicity, and any other toxicological effects likely to be elicited by a single dose.
Estimated chronic dietary exposure	The global estimate of chronic dietary exposure (GECDE) for the general population is 0.84 µg/kg bw per day, which represents 4% of the upper bound of the ADI. The GECDE for children is 2.85 µg/kg bw per day, which represents 14% of the upper bound of the ADI.
Estimated acute dietary exposure	Acute dietary exposure was not estimated because the Committee concluded that it was not necessary to establish an ARfD.
Residue definition	The Committee reconfirmed diflubenzuron as the marker residue (MR) and the ratio of the MR to the total radioactive residue (TRR) of 0.9 established at its 81st meeting.
Maximum residue limits	The Committee recommended a maximum residue limit (MRL) in salmon of 10 µg/kg in muscle+skin in natural proportions.

Ethion (acaricide)

Acceptable daily intake	The ADI of 0–0.002 mg/kg bw established by the Committee at the 85th meeting *(2)* remains unchanged.
Acute reference dose	The ARfD of 0.02 mg/kg bw established by the Committee at the 85th meeting remains unchanged.
Estimated dietary exposure	No dietary exposure assessment could be conducted.
Maximum residue limits	The Committee concluded that it would not be possible to recommend MRLs with the available data.

Flumethrin (type II pyrethroid insecticide)

Acceptable daily intake	The ADI of 0–0.004 mg/kg bw established by the Committee at the 85th meeting (2) remains unchanged.
Acute reference dose	The ARfD of 0.005 mg/kg bw established by the Committee at the 85th meeting remains unchanged.
Estimated dietary exposure	No dietary exposure assessment was conducted because no MRLs were recommended.
Maximum residue limits	The Committee concluded that it would not be possible to recommend MRLs with the available data.

Fosfomycin (broad-spectrum antimicrobial)

Acceptable daily intake	In the absence of a NOAEL and information required to determine an overall microbiological ADI (mADI), the Committee was unable to establish an ADI for fosfomycin.
Acute reference dose	The Committee established an ARfD of 0.08 mg/kg bw based on microbiological effects (specifically, disruption of the intestinal colonization barrier).

Estimated dietary exposure	No dietary exposure assessment could be conducted.
Maximum residue limits	The Committee concluded that no MRLs can be recommended with the available data.

Halquinol (broad-spectrum antimicrobial)

Acceptable daily intake	The Committee established an ADI of 0–0.2 mg/kg bw, based on histopathological changes in the kidney, accompanied by increases in absolute and relative renal weight in a 1-year chronic toxicity study in rats, applying a safety factor of 100 (10 for interspecies variability and 10 for intraspecies variability).
Acute reference dose	The Committee established an ARfD of 0.3 mg/kg bw, based on a NOAEL of 30 mg/kg bw for clinical signs in dams observed in a developmental toxicity study in mice, with application of a safety factor of 100 (10 for interspecies variability and 10 for intraspecies variability).
Estimated chronic dietary exposure	The GECDE for the general population is 5.9 µg/kg bw per day, which represents 3% of the upper bound of the ADI. The GECDE for children is 6.9 µg/kg bw per day, which represents 3.4% of the upper bound of the ADI.
Estimated acute dietary exposure	The GEADE was comparable for children and adults, being 2–224 µg/kg bw per day, which represents 0.5–75% of the ARfD.
Residue definition	The MR is the sum of 5-chloroquinolin-8-ol (5-CL), 5,7-dichloroquinolin-8-ol 5,7-DCL (5,7-DCL) and their glucuronide metabolites: 5-CLG (expressed as 5-CL equivalents) and 5,7-DCLG (expressed as 5,7-DCL equivalents).
Maximum residue limits	The Committee recommended MRLs in swine of 40 µg/kg for muscle, 350 µg/kg for skin+fat, 500 µg/kg for liver and 9000 µg/kg for kidney.

Recommended MRLs

Species	Muscle (µg/kg)	Skin+fat (µg/kg)	Liver (µg/kg)	Kidney (µg/kg)
Swine	40	350	500	9000

Ivermectin (broad-spectrum antiparasitic agent)

Acceptable daily intake	The ADI of 0–10 µg/kg bw established by the Committee at the 81st meeting *(1)* remains unchanged.
Acute reference dose	The ARfD of 0.2 mg/kg bw established by the Committee at the 81st meeting remains unchanged.
Estimated chronic dietary exposure	The Committee established a GECDE for the general population of 0.41 µg/kg bw per day, which represents 4% of the upper bound of the ADI. The Committee established a GECDE for children of 0.59 µg/kg bw per day, which represents 5.9% of the upper bound of the ADI.
Estimated acute dietary exposure	The Committee established a GEADE for the general population of 87 µg/kg bw per day, which represents 43% of the ARfD, from consumption of cattle muscle, and of 1.1 µg/kg bw, which represents 0.6% of the ARfD, from consumption of sheep muscle. The Committee established a GEADE for children of 82 µg/kg bw per day, which represents 41% of the ARfD, from consumption of cattle muscle and of 1.0 µg/kg bw, which represents 0.5% of the ARfD, from consumption of sheep muscle.
Residue definition	The MR in sheep, pigs and goats is ivermectin B1a (H2B1a, or 22,23-dihydroavermectin B1a).
Maximum residue limits	The Committee established MRLs for sheep, pigs and goats of 20 µg/kg for fat, 15 µg/kg for kidney, 15 µg/kg for liver and 10 µg/kg for muscle.

Recommended MRLs

Species	Fat (µg/kg)	Kidney (µg/kg)	Liver (µg/kg)	Muscle (µg/kg)
Sheep, pigs and goats	20	15	15	10

Selamectin (broad-spectrum antiparasitic agent)

Acceptable daily intake	The Committee established an ADI of 0–0.01 mg/kg bw, based on a NOAEL of 1 mg/kg bw per day for a reduction in serum cholesterol and triglycerides, alterations in haematology parameters, and increased liver and uterus/cervix weights in females at 5 mg/kg bw per day in a 1-year study in rats, with application of a safety factor of 100 (10 for interspecies variability and 10 for intraspecies variability).
Acute reference dose	The Committee established an ARfD of 0.4 mg/kg bw, based on a NOAEL of 40 mg/kg bw per day for malformations observed in developmental toxicity studies in rats at 60 mg/kg bw per day, with application of a safety factor of 100 (10 for interspecies variability and 10 for intraspecies variability).
Estimated dietary exposure	No dietary exposure assessment was conducted because an MRL could not be recommended.
Maximum residue limits	The Committee concluded that no MRLs can be recommended with the available data.

Sisapronil (ectoparasiticide)

No additional data were submitted. As a result, the ADI and MRLs remain unestablished.

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2. FAO/WHO. Evaluation of veterinary drug residues in food (Eighty-fifth report of the Joint FAO/WHO Expert Committee on Food Additives), WHO Technical Report Series, No. 1008. Geneva: Food and Agriculture Organization of the United Nations and the World Health Organization; 2018 (https://www.who.int/foodsafety/publications/jecfa-reports/en/).

This volume contains monographs prepared at the eighty-eighth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), which met in Rome, Italy, from 22 to 31 October 2019.

The toxicological monographs in this volume summarize data on the veterinary drug residues that were evaluated toxicologically by the Committee: diflubenzuron, fosfomycin sodium, halquinol and selamectin. Annexed to the report is a summary of the Committee's recommendations on these and other drugs discussed at the eighty-eighth meeting, including acceptable daily intakes (ADIs), acute reference doses (ARfDs) and proposed maximum residue limits (MRLs).

This volume and others in the WHO Food Additives Series contain information that is useful to those who produce and use food additives and veterinary drugs, and those involved with controlling contaminants in food, government and food regulatory officers, industrial testing laboratories, toxicological laboratories and universities.

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