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Prepared by the eighty-fifth 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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PREFACE

The monographs contained in this volume were prepared at the eighty-fifth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), which met at WHO headquarters in Geneva, Switzerland, on 17–26 October 2017. These monographs summarize the data on the safety of residues in food of selected veterinary drugs reviewed by the Committee.

The eighty-fifth report of JECFA has been published by WHO as WHO Technical Report No. 1008. 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 J. Odrowaz, Toronto, Canada. 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Many unpublished proprietary reports are submitted to the Committee by various producers of the veterinary drugs under review and in many cases represent the only data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.

These monographs were prepared based on the evaluation of the original studies and the dossier provided by the sponsor(s) of the compound, of the relevant published scientific literature and of the data submitted by Codex members. When found consistent with the data of the original study, the monographs may contain parts of the text and tables of the dossier submitted by the sponsor(s), but not the sponsor(s)' conclusions. These monographs and their conclusions are based on an independent review of the available data and do not constitute an endorsement of the sponsor(s)' position.

The designations employed and the presentation of the material in this publication do not imply the expression of any opinion whatsoever on the part of the World Health Organization concerning the legal status of any country, territory, city or area or its authorities, or concerning the delimitation of its frontiers or boundaries. The mention of specific companies or of certain manufacturers' products does not imply that they are endorsed or

recommended by the World Health Organization in preference to others of a similar nature that are not mentioned.

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

Amoxicillin (addendum)

First draft prepared by

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1. Explanation

Amoxicillin (Chemical Abstracts Service [CAS] No. 26787-78-0; Fig. 1) is a moderate spectrum, semi-synthetic β-lactam aminobenzylpenicillin antimicrobial agent used for the treatment or control of bacterial infections in animals and humans. It exerts bactericidal effects by inhibiting the transpeptidase that catalyses the cross-linking of bacterial cell wall peptidoglycan. It is available as the sodium salt and the trihydrate salt.

Amoxicillin is categorized as a highly important antimicrobial agent in the World Health Organization (WHO) list of Critically Important Antimicrobials for Human Medicine (WHO, 2017).

Amoxicillin has been previously evaluated by the Committee at its seventy-fifth meeting (Annex 1, reference *208*). A microbiological acceptable daily intake (mADI) of 0–0.007 mg/kg body weight (bw) was established. The toxicological data available at that time did not allow for the establishment of a toxicological acceptable daily intake (ADI). However, the Committee considered that the mADI would be protective for allergenicity, which was considered to be the most sensitive toxicological end-point. The Committee recommended

maximum residue limits (MRLs) for amoxicillin in cattle, sheep and pig tissue of 50 µg/kg and in cattle and sheep milk of 4 µg/L, the same as those established for benzylpenicillin and procaine penicillin at the thirty-sixth and fiftieth Joint FAO/WHO Expert Committee on Food Additives (JECFA) meetings (Annex 1, references *91* and *134*).

The Committee did not calculate an estimated daily intake for amoxicillin at the previous meeting owing to the small number of quantifiable residue data points. Using the model diet of 300 g muscle, 100 g liver, 50 g kidney, 50 g fat and 1.5 L milk with the MRLs recommended above, the theoretical maximum daily intake is 31 µg/person per day, which represents 74% of the upper bound of the ADI.

The Committee evaluated amoxicillin at the present meeting at the request of the Twenty-third Meeting of the Codex Committee on Residues of Veterinary Drugs in Foods (CCRVDF; FAO/WHO, 2016). The CCRVDF requested that JECFA recommend MRLs in finfish muscle plus skin in natural proportion.

A limited set of data was submitted to the present Committee. The data package did not include information upon which to base an evaluation for a toxicological ADI or for biochemical or microbiological effects.

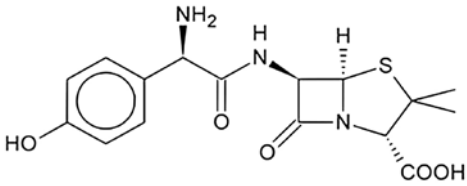
The Committee conducted a literature search in PubMed (2011–2017). The following search string was used: "Amoxicillin/adverse effects" [Mesh] OR "Amoxicillin/contraindications" [Mesh] OR "Amoxicillin/poisoning" [Mesh] OR "Amoxicillin/toxicity" [Mesh]) OR ((Amoxycillin [TIAB] OR Hydroxyampicillin [TIAB] OR Amoxicilline [TIAB] OR "BRL-2333" [TIAB] OR "BRL 2333" [TIAB] OR BRL2333 [TIAB] OR Clamoxyl [TIAB] OR Penamox [TIAB] OR Trimox [TIAB] OR Wymox [TIAB] OR Actimoxi [TIAB] OR Amoxic [TIAB]) AND ("adverse effect" [TIAB] OR "adverse effects" [TIAB] contraindication* [TIAB] OR poison* [TIAB] OR toxicit* [TIAB] OR toxic* [TIAB] OR "toxicity" [Subheading] OR "Toxicity Tests" [Mesh] OR "Poisons" [Mesh] OR "Poisons" [Pharmacological Action] OR "Toxicology" [Mesh] OR "Poisoning" [Mesh] OR "poisoning" [Subheading] OR "Poisons" [Mesh] OR "Poisons" [Pharmacological Action] OR "Toxicology" [Mesh] OR "Poisoning" [Mesh] OR "poisoning" [Subheading] OR "contraindications" [Subheading])).

The search retrieved 327 articles, of which 22 dealt with data relevant to the present toxicological and biochemical evaluation of amoxicillin. However, none of these articles provided any new information that changed the Committee's previous evaluation.

Amoxicillin, either as amoxicillin trihydrate (CAS No. 61336-70-7) or as sodium salt, has been used extensively in humans to treat a variety of infections (e.g. otitis media, pharyngitis and tonsillitis, lower respiratory tract infections, skin and skin structure infections, urinary tract infections, chlamydial infections, *Helicobacter pylori* infections and Lyme disease). The recommended oral dose is

typically 12.5 mg/kg bw per day to 50 mg/kg bw per day for an adult (60 kg) and 20–90 mg/kg bw per day for children (<40 kg).

Fig. 1
Structure of amoxicillin



2. Biological data

2.1 Observations in humans

JECFA previously concluded that the most toxicological critical effect caused by amoxicillin is hypersensitivity (Annex 1, reference *208*). Hypersensitivity, even if rare, is the most relevant toxicological effect of amoxicillin and other β-lactam antibiotics in humans. There is limited evidence that amoxicillin is less chemically reactive with proteins to form haptens than other β-lactams. The maximum concentrations tolerated in skin-prick tests and intradermal testing of benzylpenicilloyl hapten was 0.05 µmol/L compared with 51 700 µmol/L for amoxicillin, indicating that amoxicillin has significant lower reactivity than benzylpenicillin (Torres et al., 2003). No studies have been found in the literature showing allergy reactions caused by residues of amoxicillin in food. Most allergic effects have been reported after therapeutic treatment with amoxicillin, but oral exposure to small quantities of amoxicillin in sensitized individuals has been shown to elicit allergic reactions (Annex 1, reference *208*).

The thirty-sixth meeting of the Committee based its toxicological guidance value of 30 µg/person for benzylpenicillin on only four case studies of allergy to oral exposure to residues of penicillins. Considering that amoxicillin has a much lower allergenicity than benzylpenicillin, the Committee concluded that the mADI would be sufficient to protect the consumer from residues of amoxicillin in food.

2.2 Microbiological data

The Committee revised the mADI from the value of 0–0.7 µg/kg bw, established by JECFA at its seventy-fifth meeting, to 0–1.5 µg/kg bw, based on the newly adopted colon volume of 500 mL currently used in the formula to derive the mADI.

The acute reference dose (ARfD), also based on microbiological effects on the intestinal microbiota, was determined to be 4.5 µg/kg bw.

3. Comments

3.1 Toxicological data

JECFA previously concluded that the most critical effect of amoxicillin is hypersensitivity (Annex 1, reference *208*). Hypersensitivity, even if rare, is the most relevant toxicological effect of amoxicillin and other β-lactam antibiotics in humans. Amoxicillin and other β-lactams are known to react chemically with proteins to form haptens. The limited information available suggests that amoxicillin is less chemically reactive than benzylpenicillin. The maximum tolerated concentrations of benzylpenicilloyl hapten in skin-prick tests and intradermal testing was 0.05 µmol/L compared with 51 700 µmol/L for amoxicillin, indicating that amoxicillin has significant lower reactivity than benzylpenicillin (Torres et al., 2003).

At the thirty-sixth meeting, the Committee based its toxicological guidance value of 30 µg/person for benzylpenicillin on only four case studies of allergy to oral exposure to residues of penicillins. Considering that amoxicillin has a much lower allergenicity than benzylpenicillin, the Committee concluded that the mADI would be sufficient to be protective for potential allergenicity from residues of amoxicillin in food.

3.2 Microbiological data

The Committee revised the mADI from the value of 0–0.0007 mg/kg bw established at its seventy-fifth meeting to 0–0.0015 mg/kg bw based on the newly adopted colon volume of 500 mL currently used in the formula to derive the mADI (Annex 1, reference *239*).

The ARfD, also based on microbiological effects on the intestinal microbiota, was determined to be 0.0045 mg/kg bw, rounded to 0.005 mg/kg bw.

4. Evaluation

Considering that amoxicillin has a lower allergenicity than benzylpenicillin, the Committee established the mADI of 0–0.0015 mg/kg bw, rounded to 0–0.002 mg/kg bw, based on the effects of amoxicillin on the intestinal microbiota and using the newly adopted colon content volume of 500 mL. Because the majority of amoxicillin residue levels detected in target tissue were below the lowest minimum concentration required to inhibit the growth of 50% of organisms (MIC_{50}) of any of the representative human intestinal microbiota tested, it is unlikely that resistance to amoxicillin residues would develop. The Committee also considered this ADI to be protective for potential allergenicity from residues of amoxicillin.

The Committee established an ARfD of 0.0045 mg/kg bw, rounded to 0.005 mg/kg bw, also based on microbiological effects on the intestinal microbiota. The Committee considered that this ARfD would be protective for potential allergenicity from residues of amoxicillin.

5. References

FAO/WHO (2016). Report of the Twenty-third Session of the Codex Committee on Residues of Veterinary Drugs in Foods. Houston, Texas, USA, 17–21 October 2016. Rome: Food and Agriculture Organization of the United Nations and World Health Organization, Joint FAO/WHO Food Standards Programme, Codex Alimentarius Commission; 2016 (REP16/RVDF).

WHO (2017). Critically important antimicrobials for human medicine, 5th revision. Geneva: World Health Organization.

Torres MJ, Blanca M, Fernandez J, Romano A, Weck A, Aberer W et al. (2003). Diagnosis of immediate allergic reactions to beta-lactam antibiotics. Allergy. 58(10): 961–72.

Ampicillin (addendum)

First draft prepared by

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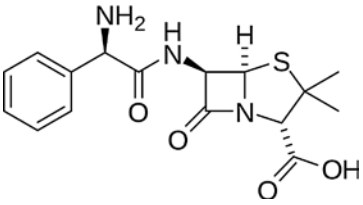
1. Explanation

Ampicillin (Chemical Abstracts Service number 69-53-4; Fig. 1) or α-aminobenzylpenicillin, is a semi-synthetic β-lactam antimicrobial agent

with an extended antibacterial spectrum, used to treat a number of bacterial infections in humans and animals caused by Gram-positive and some Gram-negative bacteria. Ampicillin has a bactericidal effect and acts as an irreversible inhibitor of transpeptidase, an enzyme important for building the bacterial cell wall. Ampicillin may have a synergistic action with aminoglycosides and with β-lactamase inhibitors.

Fig. 1
Structure of ampicillin



Ampicillin is used in several animal species, for example, cattle, sheep, pigs, fish, dogs and cats for treatment of diseases caused by different species of bacteria, e.g. *Streptococcus* spp., *Bordetella bronchiseptica*, *Pasteurella multocida*, *Trueperella pyogenes* (syn. *Arcanobacterium pyogenes*, *Corynebacterium pyogenes* and *Actinomyces pyogenes*), *Mannheimia haemolytica*, *Erysipelothrix rhusiopathiae*, *Staphylococcus aureus* and *Staphylococcus* spp. It is ineffective against β-lactamase-producing organisms.

In aquaculture, ampicillin is used for the prevention and treatment of pseudotuberculosis, vibriosis, streptococcosis and edwardsiellosis. Ampicillin trihydrate is given as an oral powder to fish at a dose of 5–20 mg/kg body weight (bw) once or twice a day for 5 days. Ampicillin sodium is administered via intramuscular injection at a single dose of 20 mg/kg bw.

In humans, ampicillin sodium or ampicillin trihydrate is mostly administered parenterally or, less often, orally at doses of 1000–2000 mg or approximately 17–33 mg/kg bw per day in adults (assumed body weight: 60 kg) to treat a number of diseases such as respiratory tract infections, urinary tract infections, meningitis, salmonellosis and endocarditis. Ampicillin has lower bioavailability than amoxicillin in humans.

Ampicillin is categorized as a highly important antimicrobial agent in the World Health Organization (WHO) Critically Important Antimicrobials (CIA) list (WHO, 2017).

Ampicillin has not previously been evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). The Committee evaluated ampicillin at the present meeting at the request of the Twenty-third Session of

the Codex Committee on Residues of Veterinary Drugs in Foods, with a view to establishing a suitable health-based guidance value (HBGV) and recommending maximum residue limits (MRLs) in finfish muscle plus skin in natural proportion (FAO/WHO, 2016). Other β-lactam antimicrobial agents, such as amoxicillin, have previously been evaluated by JECFA.

Data on ampicillin in flat fish were submitted to JECFA. The dataset did not include biochemical, toxicological or microbiological data on ampicillin. As a result, the evaluation was based on information from relevant studies identified via a literature search, most of which did not have good laboratory practice (GLP) certification.

For the biochemical, toxicological and microbiological part of the evaluation, the Committee conducted a comprehensive review of peer-reviewed scientific literature retrieved from the following publicly accessible databases: Google Scholar, PubMed, Web of Science, BioOne and ScienceDirect. The search strategy for literature relevant for the biochemical and toxicological evaluation used the keywords "Ampicillin" together with "pharmacokinetics", "kinetics", "metabolism", "excretion", "bioavailability", "toxicity", "mutagenicity", "carcinogenicity", "reproduction", "fetus", "teratogenicity", "rat", "mice", "dog", "animals", "humans", "adverse effects", "hypersensitivity", "nephrotoxicity", "kidney", "liver", "allergy", "fetus", "gastrointestinal" and the Boolean operators ("AND", "OR" and "NOT"). Some search engines did not give any relevant information. Most articles were found in PubMed. For the search ("Ampicillin/toxicity"[Mesh]) OR ("Ampicillin/pharmacokinetics"[Mesh]) NOT (Amoxicillin OR "Amoxicillin-Potassium Clavulanate combination" OR Azlocillin OR Mezlocillin OR Piperacillin OR Pivampicillin OR Talampicillin) AND (rat OR rats OR mice OR animal or animals) NOT (human OR humans OR patient or resistance), 133 articles were retrieved. For the search "Ampicillin" and "adverse effects" and "gastrointestinal", 144 articles were retrieved, but this was reduced to 51 when "adverse effects" were added. For "Ampicillin" and "allergy" and "food", 19 articles were retrieved; for "ampicillin" and "fetus/drug effects", 20 articles were retrieved; for "ampicillin hypersensitivity" and "food", 17 articles were retrieved. Many articles were to do not with ampicillin but with new compounds that were derivates of ampicillin, and many articles were case–control studies that were not relevant for this evaluation. The literature search identified 38 articles relevant for the biochemical and toxicological evaluation.

The search strategy for literature relevant for the microbiological evaluation used the keywords "ampicillin", "microbiome", "intestinal microbiota", "gut microbiota", "gut microbiome", "gastrointestinal microbiota", "gastrointestinal microbiome", "antimicrobial resistance", "susceptibility testing", "ampicillin metabolism", "excretion" and "bioavailability" as well as the genus/species and

minimum inhibitory concentration (MIC) values of specific intestinal bacteria with the Boolean operators (AND, OR and NOT).

The literature search identified 36 articles relevant for the microbiological evaluation of ampicillin.

2. Biological data

This evaluation of ampicillin relied on published review articles (and not submitted data) that did not declare GLP compliance. In addition, some studies were old and inadequately described.

2.1 Biochemical aspects

2.1.1 Absorption, distribution and excretion

(a) Mice

In a study that did not report GLP status, Adachi et al. (2002) measured plasma concentrations of ampicillin trihydrate in CB6F1-transgenic-*ras*H2 (Tg-*ras*H2) mice treated orally with 3000 mg/kg bw for 26 weeks (see section 2.2.3). Blood was sampled 1 and 3 hours after the first dose (on day 1) and the final dose (after 26 weeks). Repeated dosing over 26 weeks resulted in 2–3 times higher plasma concentrations (Table 1).

Table 1
Plasma concentrations in Tg-*ras*H2 mice treated with ampicillin trihydrate

		Plasma concentrations of ampicillin (µg/mL)	
Time after dosing		**Male**	**Female**
First dosing (day 1)	1 h	24.53 ± 0.33	23.53 ± 1.89
	3 h	2.64 ± 1.81	8.56 ± 6.29 [a]
Last dosing (26 weeks)	1 h	58.06 ± 12.07	79.57 ± 14.89
	3 h	8.56 ± 6.29*	8.48 ± 9.54

[a] The three 3-hour values appear to be incorrectly reported as they are exactly the same for females after the first dosing and for males after the final dosing
Source: Adachi et al. (2002)

In a study that did not report GLP status, a biphasic elimination was observed when mice (strain not stated) were treated intravenously with ampicillin trihydrate at a dose of 10 mg/kg bw. The half-life of the first (alpha) phase was 1.18 minutes and of the second (beta) phase was 23.20 minutes. The area under the concentration–time curve (AUC) was 296.82 ± 23.44 µg·min/mL (English, Girard & Retsema, 1976).

In another study that did not report GLP status, male and female outbred mice were treated orally with sultamicillin, an ampicillin prodrug that is hydrolysed to ampicillin and sulbactam. The half-life of ampicillin was 1.12 hours, and the AUC was 4.39 ± 0.40 μg·h/mL (English, Girard & Haskell, 1984).

(b) Rats

In a pre-GLP study, Acred et al. (1962) examined the distribution of ampicillin sodium over 24 hours in male rats (strain not stated; n = 10/group) orally treated with a single dose of ampicillin at 100 mg/kg bw. Animals were killed at 0.5, 1, 2, 4, 12 and 24 hours, and organs, tissues and excreta were examined.

Ampicillin was more-or-less evenly distributed in tissues. Maximal concentrations (μg/g wet weight) were measured in liver, spleen, kidneys, lungs, stomach, small intestine, carcass and serum at 0.5 hours, with the highest concentrations in liver and kidneys (15.82 and 13.22 μg/g, respectively). Concentrations continued to increase at 2 and 4 hours in caecum, colon and faeces. Concentrations of 4.42 μg/g were found in serum, 2.22 μg/g in spleen and 6.17 μg/g in lungs at 0.5 hours. Less than 0.09 μg/g was found 24 hours after treatment in all tissues, except for the small intestine and colon, and in faeces. In urine, the concentration increased gradually to 6.63% and 3.7% of the administered dose at 12 and 24 hours, respectively. Mean recovery of the administered dose in all tissues was 65%, 58%, 45%, 43%, 9.6% and 4.3% at 0.5, 1, 2, 4, 12 and 24 hours, respectively, indicating that ampicillin is excreted quickly in rats (Acred et al., 1962).

In a study that did not report GLP status, fasted male outbred rats were orally treated with ampicillin trihydrate at 10 or 12 mg/kg bw. The oral bioavailability was 23.0% (calculated as $[AUC_{oral}] / [AUC_{i.v.}] \times 100$) with an oral maximum concentration (C_{max}) of 0.86 ± 0.21 μg/mL and AUC_{oral} of 1.80 ± 0.01 μg·h/mL. The highest tissue concentrations were found in liver and kidney, followed by plasma, lung, spleen and muscle.

In male rats treated orally with sultamicillin at a dose of 20 mg/kg bw, the half-life for ampicillin was 0.75 hours, the AUC was 2.76 ± 0.11 μg·h/mL and the oral bioavailability was 56.7%.

In conclusion, oral absorption of ampicillin administered as a prodrug is increased 2.5-fold compared with oral absorption of ampicillin trihydrate (English, Girard & Haskell, 1984).

2.1.2 Metabolism and excretion

(a) Rats

In a study intended to evaluate a new analytical method (with limited reporting, including GLP status), Haginaka et al. (1987) treated male Wistar rats (n = 3)

intravenously with ampicillin sodium at a dose of 100 mg/kg bw. Blood samples were taken after 0, 20, 40, 60, 120 and 240 minutes and plasma samples were prepared. Bile and urine were sampled directly from the bile duct or ureter and analysed by high-performance liquid chromatography (HPLC) with pre- and post-column derivatization.

The metabolites (5*R*,6*R*)-ampicilloic acid and its epimer (5*S*,6*R*)-ampicilloic acid, and ampicillin piperazine-2,5-dione were found in plasma, bile and urine. Plasma levels of the metabolites were less than 0.5 µg/mL. Excretion in bile and urine was 75.6% of the administered intravenous dose, of which 69.5% was unchanged ampicillin and 6.1% was metabolites.

The AUC was 5.89 ± 2.68 mg·min/mL, the mean residence time was 59.3 ± 5.7 minutes, the steady state volume was 1130 ± 415 mL/kg and the clearance (CL_t) was 19.4 ± 8.2 mL/min per kg.

(b) Rabbits

In a pre-GLP study, Acred et al. (1962) treated rabbits (n = 5/group) orally with 100 mg/kg bw of ampicillin. The highest concentration in blood, 2.16 µg/mL, was measured after 1 hour; this gradually decreased to 0.06 µg/mL after 4 hours.

(c) Dogs

In a pre-GLP study, Acred et al. (1962) treated male dogs (n = 5/group) orally with 20 mg/kg bw of ampicillin. Blood samples were collected every 30 minutes for up to 5.5 hours after administration and then at 24 hours after administration. The highest concentration in serum, 7.23 µg/mL, was measured after 1 hour. After 5.5 hours, 0.11 µg/mL was detected; none was detected after 24 hours.

In male beagle dogs treated orally with 20 mg/kg bw of sultamicillin, the half-life for ampicillin was 0.98 hours and the AUC was 19.32 ± 2.06 µg·h/mL (English, Girard & Haskell, 1984).

For a summary of pharmacokinetic parameters of ampicillin in laboratory animals, see Table 2.

(d) Humans

For a summary of pharmacokinetic parameters of ampicillin in humans, see Table 3.

In a pre-GLP study, Swahn (1975) administered a dose of 500 mg of ^{35}S-labelled ampicillin (radio purity: 97%; 55.5 × 10^4 Bq) to healthy men (42–56 years old) via gastrointestinal tubes inserted through the nose prior to the experiment (n = 6) and intravenously (n = 1). Polyethylene glycol (PEG 4000) acted as a nonabsorbable marker. Blood samples were collected every 10 minutes

Table 2
Summary of pharmacokinetic parameters of ampicillin in orally treated laboratory animals

Species	Test substance	Bioavailability (%)	Time (h)	Dose (mg/kg bw)	T_{max} (h)	$t_{½}$ (h)	C_{max} (µg//mL)	AUC (µg·h/mL)	Reference
Mice	Ampicillin	–	4	20	1	1.12	–	4.39 ± 0.40	English, Girard & Haskell (1984)
Rats (male)	Ampicillin	–	24	100	0.5	–	4.42	–	Acred et al. (1962)
Rats (male)	Ampicillin trihydrate	23	24	10–12	0.5	0.75	0.86 ± 0.21	1.80 ± 0.01	English, Girard & Haskell (1984)
Rats (male)	Sultamicillin	56.7	4?	20	–	0.75	–	2.76 ± 0.11	English, Girard & Haskell (1984)
Rabbits	Ampicillin	–	–	100	1	–	2.16	–	Acred et al. (1962)
Dogs (male)	Ampicillin	–	24	20	1	–	7.23	–	Acred et al. (1962)
Dogs (male)	Sultamicillin	–	6	20	–	0.98	–	19.32 ± 2.06	English, Girard & Haskell (1984)

AUC: area under the concentration–time curve; bw: body weight; C_{max}: maximum plasma concentration; $t_{½}$: half-life; T_{max}: time to reach the maximum concentration

during the first hour, every 15 minutes during the second hour, every 60 minutes for up to 6 hours and then once at 24 hours. When possible, urine was sampled every 90 minutes during the first 6 hours and for up to 7 days. The cumulative radioactivity was measured after 3 hours and 1 and 7 days. Faeces were collected over the 7 days, and the cumulative radioactivity was measured after 7 days. Radioactivity was analysed in all samples.

At the time to reach peak plasma concentration (T_{max}), 1.7 hours (range: 1–3 h), the total plasma volume contained 2–4.4% of the administered dose. The half-life in plasma was 94 minutes (range: 75–107 minutes).

Thin layer chromatography of gastrointestinal samples found that more than 95% of the radioactivity in the samples was associated with the parent compound. Recovery of radioactivity in urine after oral dosing was 24 ± 11% after 3 hours, 43 ± 15% after 1 day and 44 ± 15% after 7 days. In urine, 87% of the excreted radioactivity was the parent compound ampicillin. Recovery of the radioactive dose in faeces was 39 ± 19% after 7 days. Recovery after intravenous dosing was 78% after 3 hours and 87% after 1 and 7 days in urine, and 5% after 7 days in faeces.

In a pre-GLP study, 17 humans (10 males, 7 females), each received a capsule containing 250 (n = 10), 500 (n = 7), 750 (n = 7) or 1000 (n = 10) mg of ampicillin trihydrate after overnight fasting. No information about the time between doses was given. Serum concentrations were measured 0.5, 1,

Table 3
Pharmacokinetic parameters of ampicillin sodium, ampicillin and metabolites in humans

Formulation	Route	Excretion (%)	Time (h)	Dose (mg/kg bw)	T_{max} (h)	$t_{½}$ (h)	C_{max} (µg//mL)	AUC (µg·h/mL)	Reference
Ampicillin	Oral	43	0–8	500	–	–	2–4	8.2 (P) to 12.6 (nonP) [a]	Philipson (1977)
Ampicillin	i.v.	79	0–8	500	–	–	44	18.6 (P) to 28.3 (nonP) [a]	
Ampicillin trihydrate	Oral (fasting)	37.1 ± 12.1 (urine)	0–8	500	1.86 ± 0.3	–	5.9 ± 2.1	19.8 ± 5.5	Eshelman & Spyker (1978)
	Oral (non-fasting)	26.8 ± 8.3 (urine)	0–8	500	2.40 ± 0.41	–	4.6 ± 1.6	13.7 ± 4.9	
Ampicillin	Oral	25.1 (urine)	0–8	500	–	–	–	–	Haginaka & Wakai (1987)
		3.29 ((5*R*,6*R*)-ampicilloic acid) (urine)							
		1.82 ((5*S*,6*R*)-ampicilloic acid) (urine)							
		0.37 (ampicillin piperazine-2,5-dione) (urine)							
Ampicillin trihydrate	Oral (fasting)	33 (urine)	0–6	250	1	–	2.19	–	Knudsen, Rolinson & Stevens (1961)
		25 (urine)	0–6	500	2	–	3.8	–	
		28 (urine)	0–6	750	2	–	5.1	–	
		33 (urine)	0–6	1 000	2	–	6.79	–	
Ampicillin	Oral (fasting)	25.8 ± 17.1 (urine)	0–12	500	–	–	–	–	Cole, Kenig & Hewitt (1973)
Ampicilloic acid	Oral	6.7 ± 5.9 (urine)	0–12	500	–	–	–	–	
Ampicillin	Oral (fasting)	43.3 ± 18.2 (urine)	0–12	250	–	–	–	–	
Ampicilloic acid	Oral	10.8 ± 6.4 (urine)	0–12	250	–	–	–	–	
Ampicillin	Oral (non-fasting)	26.4 ± 9.5 (urine)	0–12	500	–	–	–	–	Klein & Finland (1963)
	Oral (fasting)	25.4 ± 11.7 (urine)	0–12	500	–	–	–	–	
	Oral (fasting)	24.4 ± 5.8 (urine)	0–12	1 000	–	–	–	–	
	Oral (non-fasting)	22.4 ± 7.3 (urine)	0–12	1 000	–	–	–	–	
^{35}S-Ampicillin	Oral (fasting)	24 ± 11 (urine)	0–3	500	1–3	1.57 (94 mins (range: 75–107 min)	5.8	–	Swahn (1975)
		43 ± 15 (urine)	0–24	500	–	–	–	–	
		44 ± 15 (urine)	0–168	500	–	–	–	–	

Formulation	Route	Excretion (%)	Time (h)	Dose (mg/kg bw)	T_{max} (h)	$t_{½}$ (h)	C_{max} (µg//mL)	AUC (µg·h/mL)	Reference
^{35}S-Ampicillin	Oral (fasting)	39 ± 19 (faeces)	0–168	500	–	–	–	–	
Ampicillin trihydrate	Oral treatment with 25 mL water	66.4 ± 24.9 (urine)	0–8	500	–	–	–	–	Welling et al. (1977)
	Oral treatment with 250 mL water	82.8 (urine)	0–8	500	–	–	–	–	
	Oral treatment with high carbohydrate meal	27.2 ± 17.5 (urine)	0–8	500	–	–	–	–	
	Oral treatment with high fat meal	42.8 (urine)	0–8	500	–	–	–	–	
	Oral treatment with high protein meal	21.8 ± 5.7 (urine)	0–8	500	–	–	–	–	
Ampicillin	Oral (fasting)	26.6 ± 4.0 (urine)	0–2	250	–	–	–	–	Saito, Kato & Murakami (1975)
		21.0 ± 3.8 (urine)	2–4	250	2	–	2.61 ± 0.31	–	
	Oral (non-fasting)	13.2 ± 3.2 (urine)	0–2	250	–	–	–	–	
		22.2 ± 2.0 (urine)	2–4	250	2	–	1.45 ± 0.12	–	
	Oral (fasting)	43.6 (urine)	0–4	250	–	–	–	–	
	Oral (non-fasting – Meal A)	37.3 (urine)	0–4	250	–	–	–	–	
	Oral (fasting)	30 (urine)	0–14	250	–	–	–	–	
	Oral (non-fasting – Meal B)	40 (urine)	0–14	250	–	–	–	–	

AUC: area under the concentration–time curve ; bw: body weight; C_{max} : maximum concentration; i.v.: intravenous; nonP: nonpregnant; P: pregnant; $t_{½}$: half-life; T_{max}: time to reach C_{max}

[a] Bioavailability was calculated from these values: oral AUC/i.v. AUC = 45.6% for pregnant (P) women and 48.1% for nonpregnant (nonP) women.

2, 4 and 6 hours after dosing, with breakfasts consumed after the 2-hour blood sample was taken. Urine was sampled 6 hours after dosing.

The C_{max} for the lowest dose group was 2.19 μg/mL after 1 hour, and 3.8, 5.1 and 6.79 μg/mL after 2 hours at 500, 750 and 1000 mg, respectively.

Ampicillin was rapidly absorbed after oral administration., with a maximum concentration in serum after 1 to 2 hours. Approximately 30% of the administered dose was excreted in urine (Knudsen, Rolinson & Stevens, 1961).

Eshelman & Spyker (1978) investigated the effect of food on pharmacokinetic parameters in a double blind crossover study comparing absorption rates following oral administration of 500 mg ampicillin capsules by fasting and non-fasting individuals. Study participants (n = 16 males) fasted until 3 hours after administration. Sampling was conducted over 8 hours.

Fasting resulted in a faster absorption rate (0.34 ± 0.36 versus 1.43 ± 0.61 hours in non-fasting study participants); higher C_{max} (5.9 ± 2.1 versus 4.6 ± 1.6 μg/mL in non-fasting study participants); shorter T_{max} (1.86 ± 0.3 versus 2.40 ± 0.41 hours); higher urine recovery (37.1 ± 12.1% versus 26.8 ± 8.3%); and higher AUC (19.8 ± 5.5 versus 13.7 ± 4.9 h·μg/mL). GLP compliance was not reported.

Saito, Kato & Murakami (1975) compared pre- and postprandial levels of ampicillin in healthy males administered ampicillin 3 times/day at a dose of 250 mg and given different Japanese food at mealtimes but the same breakfast. Total blood levels and urinary excretion differed depending on the diet. The maximum blood concentration was decreased after each dose. Pre- and postprandial blood levels 2 hours after first dosing were 2.61 ± 0.31 μg/mL and 1.45 ± 0.12 μg/mL, respectively. Blood levels and urinary excretion of ampicillin differed depending on the diet. Preprandial urinary excretion after the first dose was 26.6 ± 4.0% at 0–2 hours and 21.0 ± 3.8% at 2–4 hours; postprandial urinary excretion was 13.2 ± 3.2% at 0–2 hours and 22.2 ± 2.0% at 2–4 hours. The mean total urinary excretion after three doses a day measured 0–4 hours after each administration, was 43.6% and 37.3% after pre- and postprandial administration with a meal of sashimi and rice, fried shrimp and rice. The mean total urinary excretion after the third dose sampled over 0–14 hours measured 30% and 41% after pre- or postprandial administration, respectively after a meal of chirashi-zushi, roast chicken and rice.

Philipson (1977) investigated the bioavailability of ampicillin in humans during and after pregnancy. Pregnant women (n = 26) with a normal pregnancy were treated orally 4 times/day with 0.5 g of ampicillin for 10 days. Concentrations in plasma and urine were measured after Dose A (0.5 g given as a first single intravenous dose); after Dose B (0.5 g given orally at least 1 week after the completion of therapy); after Dose C (0.5 g given as one single intravenous dose 3–12 months after birth); and after the last dose, D (0.5 g given at least 1 week after Dose C). Each woman was her own control during or after pregnancy. Blood was

sampled after 10, 20, 30, 45 minutes and 1, 2, 3, 5 and 8 hours after administration of Dose A and C and after 1, 2, 3, 5 and 8 hours after administration of Dose B and D. Urine was collected 3 and 8 hours after each dose. Groups B and D fasted before treatment. A disk agar diffusion method with test strains of *Sarcina lutea* for plasma and *Staphylococcus aureus* for urine was used for analyses.

Bioavailability, calculated as the mean AUC after intravenous and oral treatment, was 45.6 ± 20.2% in pregnant women and 48.1 ± 19.3% in nonpregnant women (see Table 3). The dose recovered in urine after intravenous dosing was approximately 79% for both pregnant and nonpregnant women, and after oral dosing, approximately 42% and 44% in pregnant and nonpregnant women, respectively.

The metabolism of ampicillin was investigated in two studies, neither of which reported GLP status. An HPLC method with post-column alkaline degeneration for analysing the metabolites in human urine was developed. One person was treated with 500 mg ampicillin, and their urine was sampled up to 8 hours after administration.

The total urinary excretion was approximately 30% of the given dose. Of the total dose excreted in urine, 3.29% consisted of (5*R*,6*R*)-ampicilloic acid; 1.82% of its epimer, (5*S*,6*R*)-ampicilloic acid; 0.37% of ampicillin piperazine-2,5-dione; and 25.1% of ampicillin (Table 3). The mean residence time from administration to excretion in urine was approximately 2–3.5 hours for parent compound and metabolites (Haginaka & Wakai, 1987).

In a study that did not report GLP status, Cole, Kenig & Hewitt (1973) administered ampicillin orally at a dose of 250 mg (n = 10) or 500 mg (n = 6) to humans (21–45 years old, both sexes) after overnight fasting. Because ampicillin is metabolized to some extent (7–11%) by β-lactamases, which hydrolyse the β-lactam ring to the microbiologically inactive penicilloic acid, both ampicillin and ampicilloic acid were measured in urine.

Recovery of ampicillin in urine over 0–12 hours was 43.3 ± 18.2% and 25.8 ± 17.1% for the lower and higher dose, respectively; recovery of ampicilloic acid was 10.8 ± 6.4% and 6.7 ± 5.9%, respectively. A total of 54.0 ± 19.6% of the low dose and 32.5 ± 19.0% of the high dose of the administered ampicillin were recovered.

In a study that did not report GLP status, the urinary recovery of ampicillin was measured after an oral dose of 278 mg ampicillin given to healthy students (n = 9) after overnight fasting (Rozencweig, Staquet & Klastersky, 1975). Over 0–6 hours, 56% of the administered dose was excreted in urine, an amount that was higher than reported elsewhere (25–51%).

The effect of food on absorption and excretion of single oral doses of 500 or 1000 mg of ampicillin, administered either immediately after breakfast or after overnight fasting to six males/dose, was examined in a study that did not report GLP status. Urine was sampled up to 12 hours after administration. Total

recovered ampicillin in urine in non-fasting men was 26.4 ± 9.5% at 500 mg and 22.4 ± 7.3% at 1000 mg; and in the fasting men was 25.4 ± 11.7% at 500 mg and 24.4 ± 5.8% at 1000 mg. This study found no difference in excretion in urine between administration after fasting and not fasting (Klein & Finland, 1963).

Welling et al. (1977) studied the excretion of ampicillin after treatment with 500 mg ampicillin trihydrate in fasting and non-fasting individuals (n = 3/sex per group). The study participants fasted overnight before each treatment. The test substance was given with a high carbohydrate, high fat or high protein meal or with 25 or 250 mL of water.

Recovery in urine over 8 hours was 27.2% ± 17.5% after a high carbohydrate meal; 42.8% [1] after a high fat meal; 21.8 ± 5.7% after a high protein meal; 66.4 ± 24.9% after 25 mL water; and 82.8% [1] after 250 mL water. Serum levels in participants given 500 mg ampicillin trihydrate with 25 or 250 mL water were approximately 50% higher than in those given ampicillin trihydrate with food.

These kinetic parameters were similar to the Eshelman & Spyker (1978) study findings. Absorption and excretion rates of amoxicillin were faster and higher than of ampicillin in humans. The serum levels of amoxicillin after non-fasting study participants were nearly the same as in ampicillin-treated fasted study participants.

Ampicillin is known to cross the placenta. It is also found in breast milk, and therefore may be ingested during feeding (Nathanson et al., 2000).

2.1.3 Protein binding

In a pre-GLP study, the protein binding (mean values) of ampicillin in human, bovine and equine serum was 17%, 17% and 8%, respectively (Acred et al., 1962). Brown (1964) estimated the protein binding of ampicillin in rat serum to be 5.2%. As protein binding for ampicillin is low, it circulates mainly in the free form.

2.1.4 Conclusion on the absorption, distribution, metabolism and excretion in humans and animals

Ampicillin is rapidly absorbed in mice, rats and humans (Tables 2 and 3). Ampicillin is widely distributed in tissues throughout the body. The plasma protein binding of ampicillin in humans, cattle and horses was 17%, 17% and 8%, respectively.

The metabolites found in rats after intravenous treatment were identified in humans after oral administration of ampicillin: apart from the parent

[1] Measured in only two participants so no standard deviation was reported for these groups. Other parameters were measured in three persons.

compound, the metabolites were (5*R*,6*R*)-ampicilloic acid and its epimer (5*S*,6*R*)-ampicilloic acid, and ampicillin piperazine-2,5-dione.

Excretion in humans is chiefly through the urine, with some ampicillin distributed to tissues and some excreted in faeces. Excretion in urine appears to depend on fasting state and time but not on dose. The variation between individuals is large. For nonfasting humans, mean values in different studies vary between 21.8% and 42.8% over 0–8 hours and between 22.4% and 26.4% over 0–12 hours. For fasting humans, mean values in different studies vary between 21.0% and 26.6% over 0–2 hours, 24% over 3 hours, 21.0% over 2–4 hours, 25–33% or 56% over 0–6 hours, 37.1–66.4% or 82.8% over 0–8 hours, 24.4–43.3% over 0–12 hours, 43% over 24 hours and 44% over 7 days. The variations in non-fasting study participants appear to be lower than for fasting study participants. Only one study used radiolabelled ampicillin (Swahn, 1975).

2.2 Toxicological data

2.2.1 Acute toxicity

None of the acute toxicity studies were reported as being GLP compliant.

Acute toxicity was investigated in rats and mice after subcutaneous or oral dosing with ampicillin (sodium salt) of up to 5000 mg/kg bw (Acred et al., 1962). The oral lethal median dose (LD_{50}) was greater than 5000 mg/kg bw in adult mice and rats. Intravenous administration of 2500 mg/kg bw resulted in 3/10 deaths. There were no deaths at doses of 2000 mg/kg bw, but muscle tremors, slowed respiration and mild clonic convulsions were noted in some animals.

The National Toxicology Program (NTP; 1987) cited oral LD_{50} values in mice of 15 200 mg/kg bw and in rats of 10 000 mg/kg bw. The LD_{50} in 1-day-old rats was 3300 mg and in 83-day-old rats was 4500 mg/kg bw (NTP, 1987).

After oral doses of ampicillin at 5, 15 or 50 mg/kg per day for 3 consecutive days, 63%, 45%, and 100% of the treated rabbits died (Milhaud et al., 1976). The Committee noted that many antibiotics cause gastrointestinal problems such as enteritis and disruption of the intestinal microbiota that can lead to death in rabbits.

2.2.2 Short-term studies of toxicity

None of the short-term toxicity studies were reported as being GLP compliant.

In all the NTP (1987) studies, several mice and rats died as a result of gavage errors. In the 13-week and 104-week studies, the doses were administered 5 days/week and the dosage was subsequently recalculated on a 7 day/week basis. Ampicillin trihydrate was chosen as a model substance for the ampicillin-type

penicillins. Ampicillin trihydrate was not stable in feed and was suspended in corn oil because it is only slightly soluble in water.

(a) Mice

Mice (strain B6C3F1; *n* = 5/sex per group; 52 days old) were orally treated with ampicillin trihydrate in corn oil by gavage at doses of 0, 200, 400, 800, 1600 or 2400 mg/kg bw per day for 14 days (NTP, 1987).

The no-observed-adverse-effect level (NOAEL) was 1600 mg/kg bw per day based on body-weight loss in males and diarrhoea at 2400 mg/kg bw per day.

Mice (B6C3F1; *n* = 10/sex per group; 7 weeks old) were administered ampicillin trihydrate in corn oil, by gavage, 5 days/week for 13 weeks, at doses of 0, 250, 500, 1000, 2000 or 3000 mg/kg bw per day (representing 0, 179, 357, 714, 1429 and 2143 mg/kg bw per day for 7 days/week).

No adverse effects were reported. At 2000 and 3000 mg/kg bw, respectively, 8/10 and 7/10 mice survived compared with 9–10/10 mice in the other dose groups. The slightly higher mortality in the two highest dose groups was explained as being due to a failure in gavage techniques. Gross pathological changes and histopathological alterations were not treatment related.

The NOAEL was 2143 mg/kg bw per day, the highest dose tested (NTP, 1987).

(b) Rats

In a dose range–finding study, rats (Fischer 344/N; *n* = 5/sex per group; 52 days old) were administered ampicillin trihydrate in corn oil, by gavage, at doses of 0, 200, 400, 800, 1600 or 2400 mg/kg bw per day for 14 consecutive days.

Final mean body weights in males were decreased by 8%, 12%, 11%, 9% and 14%, respectively, compared with the control group. At 3–7% across all treated groups, the decreases in female body weights were less pronounced. Clinical signs such as diarrhoea and excessive salivation were noted immediately after dosing, but details were not reported and it appears that these clinical signs were observed only in the high-dose rats although the study described them as being dose related. There were no deaths, no dose-related gross pathological changes and no histopathological alterations in the highest dose group.

As this was a dose range–finding study with few specimens, no NOAEL was determined or conclusions reached (NTP, 1987).

Acred et al. (1962) administered ampicillin sodium orally through stomach tubes to rats (strain not stated; *n* = 12 males/group) at doses of 0, 100 or 500 mg/kg bw per day for 5 days/week (representing 0, 7, 14 and 357 mg/kg bw per day for 7 days/week) for 12 weeks. Clinical analyses of red and white blood cell counts and urine glucose and protein tests were conducted weekly.

Haemoglobin levels were measured after weeks 1, 6 and 12. At the end of the study, all rats were killed and weights of liver, spleen, kidneys, testes and adrenals were recorded. A range of organs were histopathologically examined.

No toxicity was noted and organ histology was normal. Detailed results were not reported.

Rats (F344/N) were administered ampicillin trihydrate in corn oil, by gavage, at doses of 0, 180, 370, 750, 1500 or 2 × 1500 mg/kg bw per day for 5 days/week (representing 0, 129, 264, 536, 1071 or 2143 mg/kg bw per day for 7 days/week) for 13 weeks.

A slight but not dose-related decrease in final mean body weight was seen in males (6% in the lowest dose group, reaching 9% in the highest dose group), but there was no dose-related decrease in body weight in females. Twelve rats died due to gavage techniques, 1–2 males in each group including the control group and one female at 370 and three at 2 × 1500 mg/kg bw per day. Rats in the highest dose group had diarrhoea. Gross pathological and histopathological examinations did not show any treatment-related effects.

The NOAEL was 1071 mg/kg bw per day based on diarrhoea at 2143 mg/kg bw per day (NTP, 1987).

NTP concluded that there were no dose-related effects in the two highest dose groups during the 13-week treatment. Therefore, 750 and 1500 mg/kg bw were chosen as the doses for use in the 2-year study (section 2.2.3).

In a poorly described study investigating the influences of a protein-rich diet on the neurotoxic effect of ampicillin, rats (western albino; 21 days old; n = 10 males/group) were administered ampicillin through an orogastric tube at a dose of 50 mg/kg bw per day for 3 weeks while receiving a standard diet for 3 weeks. One group received the same dose of ampicillin for 3 weeks followed by a diet fortified with high protein for 10 weeks. Propionic acid was used as a positive control.

The one dose of ampicillin tested resulted in decreased levels of the neurotransmitters noradrenaline, dopamine and serotonin in whole brain homogenates compared with controls. The effects were potentiated by the protein-rich diet. Other measured parameters, for example, glutamate and interleukin 6 levels, were not significantly increased.

As only one dose of ampicillin was administered, a NOAEL could not be determined (Bhat, Chandrul & El-Ansary, 2016).

For a summary of short-term toxicity studies with ampicillin, see Table 4.

2.2.3 Long-term studies of toxicity and carcinogenicity

The literature search retrieved three carcinogenicity studies, two in mice (26 weeks and 103 weeks) and one in rats (103 weeks).

Table 4
Summary of short-term studies of the toxicity of ampicillin

Species/strain	Route	Doses (mg/kg bw)	Treatment	Effects/NOAEL	Reference
Mice (B6C3F1)	Gavage	200, 400, 800, 1 600 or 2 400, in corn oil	14 days	Decreased bw in males; diarrhoea in males and females in the highest dose group NOAEL = 1 600 mg/kg bw	NTP (1987)
Mice (B6C3F1)	Gavage	0, 179, 357, 714, 1 429 or 2 143, in corn oil	5 days/week for 13 weeks [a]	Final mean bw decreased but not dose-dependently NOAEL = 2 143 mg/kg bw	NTP (1987)
Rats F344/N (M/F)	Gavage	0, 200, 400, 800, 1 600 or 2 400, in corn oil	14 days	Dose range–finding study. No NOAEL	NTP (1987)
Rats, strain unknown (M)	Gavage	0, 100 or 500	5 days/week for 12 weeks	No effects	Acred et al. (1962)
Rats F344/N (M/F)	Gavage	0, 129, 264, 536, 1 071 or 2 143, in corn oil	5 days/week for 13 weeks [a]	A non-dose-related decrease in mean bw and diarrhoea at the highest dose LOAEL 1 500 mg/kg bw	NTP (1987)
Rats (western albino)	Gavage	0 or 50	3 weeks	Effects on neurotransmitter levels. No NOAEL	Bhat, Chandrul & El-Ansary (2016)

bw: body weight; F: females; LOAEL: lowest-observed-adverse-effect level; NOAEL: no-observed-adverse-effect level; M: males
[a] Doses recalculated from administered 5 days/week to mg/kg bw per day for 7 days/week.

(a) Mice

In a study with no GLP statement included, transgenic (Tg-*ras*H2) CB6F1 mice (n = 15/sex per group; 8–26 weeks old over the course of the study) were orally treated with ampicillin trihydrate in 0.5% solution of carboxymethylcellulose sodium salt at doses of 0, 350, 1000 or 3000 mg/kg bw per day for 26 weeks. Non-transgenic (non-Tg) mice were treated orally with ampicillin trihydrate at 3000 mg/kg bw per day for the same length of time. A positive control group of Tg-*ras*H2 mice was also treated with a single intraperitoneal dose of 75 mg/kg bw of *N*-methyl-*N*-nitrosourea (MNU).

Ampicillin trihydrate treatment did not affect mortality in either the Tg-*ras*H2 or non-Tg mice. In the positive control group, all surviving Tg-*ras*H2 mice were killed at week 18 because seven males and four females had died of tumours by that time. Loose stools were dose dependent in mice treated with ampicillin trihydrate but not in mice treated with MNU. Body-weight gain in Tg-*ras*H2 male mice was significantly lower in all treated groups compared with controls. In treated female Tg-*ras*H2 mice, body-weight gain was significantly lower in weeks 4–26 in the two lowest dose groups and in weeks 5–26 in the highest dose group. In non-Tg mice, body-weight gain was lower than controls during weeks 1–26 in males and sporadically in females. The MNU treatment did not result in

lower body weights to the same extent as ampicillin trihydrate treatment. Relative brain and carcass weights decreased dose-dependently in treated male Tg-*ras*H2 mice only; in females, relative carcass weights decreased at 350 and 3000 mg/kg bw per day and relative brain weight decreased at 350 and 1000 mg/kg bw per day. Relative carcass weights were decreased in the male non-Tg mice but were relative brain, liver, kidney, testes and heart wrights were increased. In females, relative liver, heart and kidney weights were increased. These findings were considered to be due to a depression in body-weight gain. Dilation of caecum was dose dependent in treated Tg-*ras*H2 and non-Tg mice. No histopathological findings were associated with ampicillin trihydrate treatment. Several different tumours were found in the positive control group within the 18-week treatment period, showing that MNU was carcinogenic in Tg-*ras*H2 mice.

Ampicillin trihydrate was not carcinogenic in Tg-*ras*H2 mice and non-Tg mice treated for 26 weeks. However, no NOAEL could be determined as decreased body weights and dilation of caecum were seen in all treated groups (Adachi et al., 2002).

In a study that did not report GLP status, mice (C57BL/6N, females; XC3H/HeN MT, males; n = 50/sex per group) were administered ampicillin trihydrate in corn oil, by gavage, at 0, 1500 or 3000 mg/kg bw for 5 days/week over 103 weeks (representing 0, 1071 and 2143 mg/kg bw per day for 7 days/week).

At study end after 2 years, mean body weights in males were slightly decreased (4%) compared with controls. No mean body-weight changes were seen in females. Increased salivation and decreased activity were considered treatment related. The incidence of nonaccidental deaths was higher in treated groups, resulting in fewer treated survivors at study end. Accidental deaths due to drowning or gavage accidents were also more frequent in treated groups. However, a Kaplan–Meier survival curve showed that there was no significant difference in survival between the groups. Increases in forestomach lesions (ulcers, inflammation, hyperkeratosis, acanthosis and fungal infections) were significant but not dose related in treated mice; the lesions did not produce neoplastic responses. Non-neoplastic lesions, for example, hyperplasia (granulocytic) in the bone marrow, were more frequent in males in all treated groups than in controls: 7/45 (16%), 13/47 (28%) and 13/47 (28%) at 0, 1500 and 3000 mg/kg bw per day, respectively. No historical control data on hyperplasia in the bone marrow were available for comparison. Hyperplasia in the mandibular lymph node increased dose-dependently in female mice: 1/32 (3%), 7/37 (19%) and 9/37 (24%) at 0, 1500 and 3000 mg/kg bw per day, respectively; these increases were not seen in male mice. Control mice had a higher incidence of total primary tumours, malignant tumours and benign tumours than did treated mice, that is, there was no increase in dose-dependent incidence of neoplasms compared with controls.

In view of the hyperplasia observed in both treated groups, a NOAEL could not be determined (NTP, 1987).

(b) **Rats**

In a study that did not report GLP status, rats (F344/N, *n* = 50/sex per group, 7–8 weeks old) were treated, by gavage, with ampicillin trihydrate in corn oil for 103 weeks at doses of 0, 750 or 1500 mg/kg bw per day for 5 days/week (representing 0, 536 and 1071 mg/kg bw per day for 7 days/week).

Ampicillin trihydrate treatment did not affect mean body weights although a very slight decrease was noticed in high-dose males. Diarrhoea, chromodacryorrhoea and excessive urination were noted as treatment related. Survival rate for males and females was not significantly affected by treatment. The incidence of mononuclear cell leukaemia was significantly higher in males (control: 5/50 [10%]; low dose: 14/50 [28%]; and high dose: 13/50 [26%]), but the incidence was within historical control range, 152/1100 (13.8% or 2–28%), which was the rate of mononuclear cell leukaemia subsequently found to be a natural cause of spontaneous death in aged F344/N rats (Maronpot et al., 2016). As a result, the NTP changed the strain of rats used in subsequent carcinogenicity studies. Classification of the mononuclear cell leukaemia in three severity stages showed that advanced disease, Stage 3, was observed mostly in treated groups but was not dose related. Incidence of non-neoplastic focal cellular change of the adrenal cortex was increased in treated rats (males: 1/50, 5/50 and 7/49; females: 6/50, 12/50 and 15/49, respectively, for control, low-dose and high-dose animals). Nonmalignant pheochromocytomas of the adrenal medulla were increased in male rats at the highest dose (13/50 [26%], 12/50 [24%] and 23/49 [47%], respectively; historical control data: 247/1092 [22.3%]). The incidence of malignant pheochromocytomas was highest in low-dose males (5/50), while there was one occurrence in the control group and one in the high dose group. Hyperplasia in bone marrow was observed in both sexes (males: 7/50 [14%], 16/48 [33%] and 17/50 [34%], respectively; females: 13/50 [26%], 22/49 [45%] and 25/50 [50%], respectively). Non-neoplastic thyroid gland C-cell hyperplasia increased in low-dose males and high-dose females but not in other groups (see Table 5). Some effects (fibroadenomas, hyperplasia) were noticed in the mammary gland, but were only significant in the low dose groups. Cytoplasmic vacuolization in the liver was increased in high-dose males but not in females (data not shown). Hyperkeratosis was increased in males (6%, 14% and 20%, respectively) but not females (data not shown); acanthosis in the forestomach was also increased in males (0, 4.5% and 11%, respectively) but not in females. In addition, inflammation in the prostate was increased in high-dose males.

Table 5

Neoplastic and other effects in rats F344/N treated orally with ampicillin trihydrate for 2 years

	No. of findings and incidence per sex per dose level [a]						
	Males			Females			
Neoplastic and other effects	0 mg/kg bw per day	536 mg/ kg bw per day	1 071 mg/ kg bw per day	0 mg/kg bw per day	536 mg/ kg bw per day	1 071 mg/ kg bw per day	Historical control [b]
Haematopoietic system tumours							
Mononuclear cell leukaemia	5/50 (10%)	14/50 (28%)	13/50 (26%)	14/50 (28%)	18/50 (36%)	13/50 (26%)	152/1 100 (13.8%, 2–28%)
All leukaemia or lymphomas	6/50 (12%)	16/50 (32%)	14/50 (28%)	–	–	–	162/1 100 (14.7%, 2–28%)
Hyperplasia, bone marrow	7/50 (14%)	16/48 (33%)	17/50 (34%)	13/50 (26%)	22/49 (45%)	25/50 (50%)	–
Endocrine systems							
Adrenal cortex – Focal cellular change	1/50 (2%)	5/50 (10%)	7/49 (14%)	6/50 (12%)	12/50 (24%)	15/49 (31%)	–
Adrenal medulla							
Pheochromocytomas, nonmalignant	13/50 (26%)	12/50 (24%)	23/49 (47%)	–	–	–	247/1 092 (22.3%, 4–40%)
Pheochromocytomas, malignant	1/50 (2%)	5/50 (10%)	1/49 (2%)	–	–	–	–
Thyroid							
C-cell hyperplasia	4/50 (8%)	11/48 (23%)	7/46 (15%)	10/50 (20%)	12/49 (24%)	21/49 (43%)	–
Other organs							
Liver – cytoplasmic vacuolization	2/50 (4%)	5/49 (10%)	10/50 (20%)	–	–	–	–
Hyperkeratosis in cardiac stomach	3/48 (6%)	6/44 (14%)	9/45 (20%)	–	–	–	–
Acanthosis cardiac stomach	0/49	2/44 (4.5%)	5/45 (11%)	–	–	–	–
Reproduction							
Prostate inflammation	22/49 (45%)	27/48 (56%)	36/47 (77%)	–	–	–	–
Mammary gland fibroadenomas	–	–	–	16/50 (32%)	25/50 (50%)	19/50 (38%)	280/1 100 (25%) (7/50–19/50)
Mammary gland, hyperplasia, cystic	–	–	–	16/50 (32%)	15/50 (30%)	20/50 (40%)	–

bw: body weight; no.: number

[a] Results expressed as no. of animals with the finding / no. of animals examined and, in parentheses, as a percentage.

[b] Historical incidences in rats (F344/N and mice ($B6C3F_1$) administered ampicillin trihydrate in corn oil by gavage.

Source: NTP (1987)

A summary of the incidence of neoplasms in rats showed that treated rats had a higher number of total primary tumours than controls, but the number of rats with tumours did not differ. The total number of malignant tumours was slightly higher in treated rats: 26/22 (118%), 30/25 (120%) and 31/24 (129%) for control, low- and high-dose rats, respectively. The number of benign tumours was not higher in treated groups compared with controls: 55/33 (166%), 63/35 (180%) and 63/38 (166%), respectively. No data on haematological, clinical chemistry and urine analyses were reported.

A NOAEL could not be identified because effects were seen in both dose groups (NTP, 1987).

2.2.4 Genotoxicity

Ampicillin was tested for genotoxicity in an adequate range of assays, both in vitro and in vivo. No evidence of genotoxicity was found, and the Committee concluded that ampicillin is unlikely to be genotoxic.

Results of genotoxicity tests with ampicillin are summarized in Table 6.

2.2.5 Reproductive and developmental toxicity

(a) Reproductive toxicity

No studies on reproductive toxicity in laboratory animals were found.

(b) Developmental toxicity

Rats

The effect of ampicillin on the embryonic kidney in Sprague Dawley rats was studied ex vivo and in vivo in a study where GLP status was not reported. Pregnant rats were killed at gestation day 14. Embryonic kidneys were removed and metanephros organ culture was prepared and treated with 0, 10, 100 or 1000 µg/mL of ampicillin for 2 days after growth for 6 days. A histological investigation of the whole branching system of the ureteric bud was completed.

A dose-dependent influence on ex vivo development of renal differentiation was found with a reduction in nephron formation of up to 67% and in growth retardation of up to 25% in the highest dose group; at the lowest dose, the effect was weaker but still statistically significant. The number of glomeruli was also dose-dependently reduced in ampicillin-treated groups.

In the in vivo part of the study, pregnant Sprague Dawley rats were treated with a subcutaneous implant of ampicillin at a dose of 100 mg/kg bw per day on gestation days 11–15. After delivery, kidneys from 14-day-old pups were prepared for light microscopic and histological examination and nephron mass determination.

Table 6
Results of studies of genotoxicity with ampicillin

End-point	Test object	Concentration	Results	Reference
In vitro				
Reverse mutation–Ames test	*Salmonella typhimurium* TA98, TA100	0 (solvent control DMSO), 10, 33, 100, 333,1 000 µg/plate	Negative ±S9 [a]	NTP (1987) [b]
	TA1535, TA1537	0, 0.03, 0.10,0.30, 1.00, 2.00, 3.30 µg/ plate ±S9		
Forward mutation	Mouse lymphoma L5178Y cells	0, 313, 625, 1 250, 2 500, 5 000 µg/ mL, –S9	Negative [c]	NTP (1987) [b]
		0, 500, 1 000, 2 000, 3 000, 5 000 µg/ mL, +S9		
Sister chromatid exchange	CHO cells	50, 160, 500, 1 500 µg/mL	Negative [d]	NTP (1987) [b]
Chromosomal aberration	CHO cells	0, 250, 500, 1 000, 1 500 µg/mL	Negative [e]	
Micronucleus	Wild-type mouse embryonic fibroblasts	0, 0.035, 0.35, 3.5, 35, 350, 3 500 µg/mL	Negative [f]	Watters et al. (2009) [b]
Comet assay	Wild-type mouse embryonic fibroblasts	0, 0.035, 0.35, 3.5, 35, 350, 3 500 µg/mL	Negative [f]	Watters et al. (2009) [b]
γH2AX focus assay	Wild-type mouse embryonic fibroblasts	0, 0.035, 0.35, 3.5, 35, 350, 3 500 µg/mL	Negative [f]	Watters et al. (2009) [b]
Combining in vitro phenotypic parameters with transcriptomic data	HepG2 cells	250 µmol/L	Negative [g]	Magkoufopoulou et al. (2011)
In vivo				
Chromosomal aberration, mitotic index, cell cycle	Human lymphocytes	0, 7, 14 µg/mL	Negative [h]	Jaju, Jaju & Ahuja (1984) b
		28 µg/mL	Positive	
Sister chromatid exchange	Human lymphocytes	0, 7, 14, 28 µg/mL	Negative [h]	Jaju, Jaju & Ahuja (1984) b
		28 µg/mL	Positive	
Micronucleus	PVG rats (males)	5 g/kg bw	Negative [i]	Stemp, Pascoe & Gatehouse (1989) [b]
Chromosomal aberration	Human lymphocytes	0, 5, 10 mg/mL	Negative, mitotic index 40% [i]	Stemp, Pascoe & Gatehouse (1989) [b]
Comet assay, alkaline	Rat, Sprague Dawley	0, 25, 50, 100 mg/kg bw (saline vehicle)	Negative [j]	McNamee & Bellier (2015) [b]
		0, 500, 1 000, 2 000 mg/kg bw (corn oil vehicle)		

bw: body weight; CHO: Chinese hamster ovary; DMSO: dimethyl sulfoxide; DNA: deoxyribonucleic acid; HPRT: hypoxanthine–guanine phosphoribosyltransferase; S9: 9000 × *g* fraction from Aroclor 1254 or phenobarbital/5,6-benzoflavone–induced pretreated rat liver homogenate; SCE: sister chromatid exchange

[a] Ampicillin trihydrate was tested twice in the Ames test in *S. typhimurium* strains TA98, TA100, TA1535 and TA1537 in the presence or absence of S9 mix prepared from Aroclor 1254-induced liver cells from male Syrian hamster or male Sprague Dawley rat. Cell toxicity was found at the highest doses in all strains without metabolic activation. Cell toxicity was observed at the highest doses in TA98 and TA100 with rat S9 mix and in TA1535, TA1537 and TA98 with hamster S9 mix. Cell toxicity was also found at 2.0 µg/ plate in TA1535 and TA1537, with or without metabolic activation. No changes of revertants/ plate were found in treated groups except in the dose groups showing cell toxicity.

[b] GLP status not reported.

[c] Ampicillin trihydrate was tested twice in L5178Y mouse lymphoma cells with or without metabolic activation. Ampicillin trihydrate was dissolved in DMSO and tested at concentrations of 0, 313, 625, 1250, 2500 and 5000 µg/mL without metabolic activation. Ethyl methane sulfonate was used as a positive control. In addition, ampicillin trihydrate was dissolved in DMSO and tested at concentrations of 0, 500, 1000, 2000, 3000 and 5000 µg/mL with metabolic activation using S9 mix from liver of Aroclor 1254-induced male F344 rats. 3-Methyl cholanthrene was used as a positive control. No difference in total mutant clones, mutation frequency (mutants/106 clonable cells), relative total growth or cloning efficiency between treated and control cells were found. The positive controls showed increased mutant clones and mutation frequency, but the relative total growth was decreased and cloning efficiency normal.

[d] CHO cells were incubated with ampicillin trihydrate (50, 160, 500 or 1500 µg/mL) with and without metabolic activation. S9 mix was prepared from the liver of Aroclor 1254-induced male Sprague Dawley rats. No increase in SCE could be found after treatment with ampicillin trihydrate either with or without metabolic activation. A dose-dependent increase in SCE was found in positive controls (mitomycin C at 0.001 or 0.010 µg/mL and cyclophosphamide at 0.3 or 2.00 µg/mL) without or with metabolic activation.

Table 6 (continued)

[e] CHO cells were incubated with ampicillin trihydrate (250, 500, 1000 or 1500 µg/mL), mitomycin C (0.25 or 1.00 µg/mL) or cyclophosphamide (15 or 50 µg/mL) as positive controls in cells treated without or with metabolic activation. S9 was prepared from the liver of Aroclor 1254-induced male Sprague Dawley rats. Chromosomal aberrations were counted in 100 cells. The positive control groups had a higher and dose-dependent increased incidence of aberrations compared with ampicillin trihydrate–treated groups and the control group. No increase in chromosomal aberrations were found in ampicillin trihydrate–treated groups compared with control groups.

[f] Ampicillin sodium (0, 0.035, 0.35, 3.5, 35, 350 or 3500 µg/mL (9.4 mmol/L) was tested in vitro for mutagenicity in the γH2AX focus assay based on increases in phosphorylation of H2AX in seeded wild-type mouse embryonic fibroblasts. The result was analysed both with manual scoring of γH2AX foci and with flow cytometry in a single-cell suspension cell line L5178Y $tk^{+/-}$ mouse lymphoma cells. Ampicillin sodium did not cause any statistically significant increases in H2AX phosphorylation, analysed as γH2AX foci. The results were confirmed in a micronucleus assay and an alkaline comet assay under identical conditions, e.g. mouse embryonic fibroblasts, doses and treatment time (4 h). Results were calculated as number of micronucleated cells per 1000 non-micronucleated cells, and scored as comet tail moments. Ampicillin sodium was negative in all genotoxic end-points tested, H2AX phosphorylation, micronucleus formation and mutation frequency in the same population of L5178Y cells.

[g] Ampicillin was used as a negative control in a study combining in vitro phenotypic parameters with transcriptomic data from HepG2 cells, a human liver cancer cell line. Cells were exposed to 250 µmol/L ampicillin. DNA damage measured by the cellular levels of γH2AX foci was quantified and was not increased after ampicillin treatment compared with control. Cell cycle distribution was not affected by treatment with ampicillin for 12, 24 or 48 hours, which showed that ampicillin did not accumulate cells in S phase, G0-G1 phase or G2-M-phase in HepG2 cells. Differential expressed genes were also unaffected by treatment. Ampicillin was shown to not affect the different end-points for genotoxicity measured in HepG2 cells.

[h] Ampicillin was tested in human ($n = 2$) lymphocytes exposed for 0, 24 or 48 hours to 0, 7, 14 (corresponding to recommended plasma levels after intramuscular treatment with 0.5 and 1 g) and 28 µg/mL. The two lowest dose groups did not cause chromosomal damage, decrease mitotic index or induce a difference in cell progression. The highest dose resulted in a delay in cell turnover rate, significant non time-related chromosomal aberrations, including gaps and a decreased mitotic index. The SCE was not influenced at any dose or time point.
In a 1982 PhD thesis (described in Jaju, Jaju & Ahuja, 1984), the effects of ampicillin were investigated in vivo on lymphocyte chromosomes from 10 humans treated with ampicillin. The chromosomal aberrations were increased to 0.07 per cell ($P < 0.05$) compared with 0.04 per cell before treatment with ampicillin. This thesis was not found in the literature search.

[i] Whole blood was cultured for 48 hours in different media, before treatment with ampicillin trihydrate dissolved in water or DMSO, for 24 hours or 72 hours after culture initiation. Daunomycin was used as a positive control. Chromosomal aberrations (including or excluding gaps) were not significantly increased when cells were treated with ampicillin trihydrate at concentration ≤1 mg/mL in any of the different media. The mitotic index was reduced 25–30%, depending on the medium. One more experiment was performed where human peripheral lymphocytes were treated with 0, 5 or 10 mg/mL of ampicillin trihydrate in water. No significant increase in chromosomal aberrations up to a test concentration of 10 mg/mL and a mitotic index of 40% was found. The different culture media did not affect the results. Ampicillin trihydrate (3 and 5 g/kg bw), dissolved in sterile water for irrigation, was administered by gavage to male PVG rats. The rats were killed after 30 hours and marrow smears were prepared. Male rats were also treated with ampicillin trihydrate (5 g/kg bw; $n = 10$/group) or vehicle control ($n = 7$) 24 and 48 hours before scheduled kill and preparation of marrow smears. Two thousand immature erythrocytes were analysed per animal. No significant increases in micronucleus or difference in the proportion of immature cells in the total erythrocyte population was found. No treatment-related clastogenic effects were observed.

[j] Ampicillin trihydrate was tested for effects on DNA in an in vivo rat alkaline comet assay. Male Sprague Dawley rats (Crl:CD) were treated by gavage with ampicillin trihydrate in isotonic saline at doses of 0, 25, 50 or 100 mg/kg bw or ampicillin trihydrate in corn oil at doses of 0, 500, 1000 or 2000 mg/kg bw at 0, 24 and 45 hours. Ethyl methanesulfonate was used as the positive control. The rats were killed 3 hours after the final dose. Glandular stomach and liver cells were prepared for analysis. Histopathological examination of liver and stomach of the higher dose groups, with corn oil as vehicle, showed no cytotoxicity or abnormalities. There was no increase in % tail DNA in treated rats compared with controls at doses of ampicillin trihydrate of up to 2000 mg/kg bw.

Treatment had no effect on duration of gestation, number of pups or dam or pup weights at birth. Treatment also had no effect on pup body or kidney weights, but the number of nephrons were lower ($P < 0.001$) in treated groups. The 100 mg/kg bw dose resulted in a higher than 20% deficit in nephrons in 25% of the exposed pups. At 200 mg/kg bw, all pups had oligonephronia (renal hypoplasia; results not shown). Histopathological examination of kidneys in newborn and 14-day-old pups showed enlarged tubular segments (dilation) of focal cystic tubules or interstitial inflammation.

It was concluded that a permanent nephron deficit occurs in the fetus when dams are treated with 100 mg/kg bw of ampicillin at a certain period in gestation. As effects on kidneys were found ex vivo and in the only dose tested in vivo, it was not possible to establish a NOAEL (Nathanson et al., 2000).

After white rats (breed not specified; $n = 54$) were treated with ampicillin at doses of 250 mg/kg bw either on days 4–13 or days 15–20 (the end of the

gestation period), placental weights were much lower in treated groups than in controls (390 ± 10.5 versus 640 ± 4.3 mg). Embryonic deaths increased significantly in ampicillin-treated rats compared with controls (27.8 ± 4.2% versus 16.6 ± 1.4%; $P < 0.05$). Fetal weight was lower in treated rats than controls (2.7 ± 0.1 versus 3.0 ± 0.1 mg; $P < 0.05$), as was newborn pup weight. However, all pups achieved approximately the same body weight as the control group 20 days after parturition. No malformations were observed (Korzhova, Lisitsyna & Mikhaĭlova, 1981).

Rats (white wild type, $n = 104$ rats, weight 180–200 g) were treated with ampicillin trihydrate at an oral dose of 50 mg/rat (250–278 mg/kg bw per day) during gestation days 1–5 ($n = 8$–9 dams), days 6–11 ($n = 9$–10 dams) or days 12–17 ($n = 8$–11 dams). Two different batches of ampicillin trihydrate were tested. The control group received physiological saline ($n = 20$ dams).The only effects observed were decreased body weight of the fetuses of dams treated on gestation days 6–11 or 12–17, and reduced craniocaudal sizes in fetuses of dams treated on gestation days 12–17, measured at the 20th day of embryonal development. Effects were observed at the only dose tested but no teratogenic effects were observed (Solovev & Kovalenko, 1973).

2.3 Microbiological effects

The Committee used a decision-tree approach to determine the need to establish a microbiological acceptable daily intake (mADI) for ampicillin. The decision-tree approach, which complies with International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products guideline (VICH GL36; VICH, 2004, 2012), was adopted by the sixty-sixth JECFA (Annex 1, reference *181*).

The decision-tree approach first determines if microbiologically active ampicillin residues are entering the human colon. If no microbiologically active ampicillin 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 acceptable daily intake (ADI) is used.

The Committee evaluated in vitro MIC susceptibility testing data, in vitro continuous culture systems, in vivo human volunteer and laboratory animal studies and faecal binding biological activity and antimicrobial resistance

studies. As the sponsor did not submit any data on the susceptibility of strains to ampicillin or the effects of ampicillin on intestinal microbiota, the Committee conducted a comprehensive literature search using several publicly accessible databases (section 1). The Committee used the information and data derived from the literature search to answer the following questions in the decision-tree.

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

Yes. Ampicillin is microbiologically active against some bacterial genera and species representative of the human intestinal microbiota. The metabolites of ampicillin, that is, ampicillin piperazine-2,5-dione, (5*R*,6*R*)-ampicilloic acid and (5*S*,6*R*)-ampicilloic acid, have not been tested for microbiological activity.

Ampicillin belongs to the penicillin group of β-lactam antimicrobial agents with a broad spectrum of bactericidal activity against many Gram-positive and some Gram-negative aerobic and anaerobic bacteria (Castle, 2007). Ampicillin is used to treat and prevent infections in animals, fish and humans. Ampicillin shows good activity against Gram-positive anaerobic bacteria such as *Bifidobacterium*, *Lactobacillus*, *Peptococcus* and some *Clostridium* strains. Gram-negative anaerobic bacterial activity varies depending upon the species.

Ampicillin acts as a competitive inhibitor of the enzyme transpeptidase, which is necessary for bacterial cell wall synthesis. Inhibition of the final transpeptidation step of peptidoglycan synthesis in bacterial cell walls leads to cell lysis (Sharma, Singh & Singh, 2013).

The spectrum of activity of ampicillin may be extended by administering it with sulbactam (Kager et al., 1982). β-Lactamase is an enzyme produced by bacteria that cleaves the β-lactam ring to inactivate ampicillin. Co-administering sulbactam, a β-lactamase inhibitor, with ampicillin restores ampicillin activity against β-lactamase-producing strains.

The sponsor did not submit any data on the susceptibility of human intestinal bacteria strains to ampicillin or other data on microbiological effects of ampicillin on intestinal bacteria for the Committee to evaluate. The Committee obtained MIC values and other data for the various genera and species from published scientific literature. Published MIC data on susceptibility to ampicillin of the intestinal microbiota were difficult to evaluate as various laboratories use different MIC test methods, not always according to internationally recognized standards such as those of the Clinical and Laboratory Standards Institute (CLSI, 2012a,b, 2014). In many cases, the number of isolates tested was low ($n < 10$), and there was a lack of MIC distribution information for the isolates. In some cases, the minimum concentration required to inhibit the growth of 50% of organisms (MIC_{50}) were based on human faecal isolates from clinical infections and not from healthy donors.

Appelbaum & Chatterton (1978) determined MIC values for 265 clinical strains of anaerobic bacteria of 10 antimicrobial agents including ampicillin. Ampicillin, at a concentration of 16 μg/mL or less, was active against most of the anaerobic bacteria screened.

Sutter & Finegold (1976) tested the susceptibility of 492 anaerobic bacteria to 23 antimicrobial agents including ampicillin. Ampicillin at 16 μg/mL or less was active against many of the anaerobic bacteria tested.

Wexler et al. (1985) determined the susceptibilities of 272 clinical isolates of anaerobic bacteria to ampicillin alone and to an ampicillin–sulbactam combination. Approximately 99.6% of the clinical isolates were inhibited by 16 μg/mL of ampicillin combined with 8 μg/mL of sulbactam. The combination of sulbactam and ampicillin was much more effective than ampicillin alone against *Bacteroides fragilis*, but no different to ampicillin alone against *Fusobacterium* and other Gram-positive rods and cocci.

Snydman et al. (2017) reported similar findings for *Bacteroides* species.

Ampicillin was active against *Fusobacterium* (MIC_{50} of 2 μg/mL); *Eubacterium* (MIC_{50} of 0.12 μg/mL); *Peptostreptococcus* (MIC_{50} of 0.5 μg/mL); *Clostridium* (MIC_{50} of 0.5 μg/mL); *Bacteroides* (MIC_{50} of 16 μg/mL); and *Lactobacillus* (MIC_{50} of 0.5 μg/mL).

In a study of 13 strains of seven species of bifidobacteria, *B. animalis*, *B. bifidum*, *B. beeve*, *B. infantis*, *B. longum* and *B. pseudolongum*, antimicrobial susceptibility to ampicillin varied, with MIC values of 0.98–31.2 μg/mL (Kheadr et al., 2007). In a more expanded survey of *Bifidobacterium* species, Mättö et al. (2007) evaluated 203 bifidobacterial strains, sourced from human intestine and probiotic cultures, for susceptibility to ampicillin and six other antibiotics, using the Etest procedure. The MIC distribution for ampicillin ranged from less than 0.016 to 2 μg/mL, with a median of 0.12 μg/mL.

Lim, Huh & Baek (1993) tested 37 strains representing six species of bifidobacteria for susceptibility to ampicillin and reported variable MIC values of less than 0.19–12.5 μg/mL.

D'Aimmo, Modesto & Biavati (2007) surveyed 23 *Lactobacillus* species isolated from dairy and pharmaceutical products and reported an MIC_{50} of 0.5 μg/mL. Klare et al. (2007) determined the antimicrobial susceptibility of 473 lactic acid bacteria (*Lactobacillus*, *Pediococcus* and *Lactococcus* human isolates) encompassing 24 species and reported an MIC range of 0.063–4 μg/mL, with an MIC_{50} of 0.5–1 μg/mL for most of the *Lactobacillus* isolates.

Louie et al. (1992) surveyed 103 clinical isolates of *Enterococcus faecium* and 34 isolates of *E. faecalis* for susceptibility to ampicillin. The MIC_{50} of 8 μg/mL for *E. faecium* isolates was generally higher than for *E. faecalis* isolates, which had an MIC_{50} of 2 μg/mL.

Table 7
Summary of ampicillin MIC_{50} values for bacterial species

Bacterial species	MIC_{50} (µg/mL)	References
Bacteroides	16	Sutter & Finegold (1976); Appelbaum & Chatterton (1978); Wexler et al. (1985); Snydman et al. (2017)
Bifidobacterium	0.12	Sutter & Finegold (1976); Lim, Huh & Baek (1993); D'Aimmo, Modesto & Biavati (2007); Kheadr et al. (2007)
Escherichia coli	2	Burrows, Morton & Fales (1993); EUCAST (2017)
Eubacterium	0.12	Sutter & Finegold (1976); Appelbaum & Chatterton (1978); Goldstein et al. (2004)
Fusobacterium	2	Sutter & Finegold (1976); Appelbaum & Chatterton (1978); Wexler et al. (1985)
Peptostreptococcus	0.5	Sutter & Finegold (1976); Appelbaum & Chatterton (1978)
Clostridium	0.5	Sutter & Finegold (1976); Appelbaum & Chatterton (1978); Wexler et al. (1985)
Lactobacillus	0.5	Sutter & Finegold (1976); Appelbaum & Chatterton (1978); Klare et al. (2007)
Enterococcus	1	Louie et al. (1992); Burrows, Morton & Fales (1993); EUCAST(2017)

MIC_{50}: minimum concentration required to inhibit the growth of 50% of organisms

European Committee on Antimicrobial Susceptibility Testing (EUCAST; 2017) MIC distribution data indicated an MIC_{50} of 1 µg/mL for *E. faecalis* and 2 µg/mL for *E. faecium*. EUCAST has established a large database of ampicillin MICs.

Burrows, Morton & Fales (1993) surveyed 635 Gram-negative bacterial isolates of equine origin. They reported MIC_{50} values of 4 µg/mL for 104 *Escherichia coli* isolates; seven isolates of *Enterobacter* sp. had an MIC_{50} of 8 µg/mL. Goldstein et al. (2004) evaluated the susceptibility to ampicillin of a broad spectrum of aerobic and aerobic Gram-positive species. *Clostridium clostridioforme* had an MIC_{50} of 1 µg/mL, *C. innocuum* had an MIC_{50} of 0.125 and *C. ramosum* was more sensitive to ampicillin, with an MIC_{50} of 0.06 µg/mL. The MIC_{50} for *Lactobacillus* spp. ranged from 0.03 to 1 µg/mL, and for *Eubacterium* sp. from 0.03 to 0.25 µg/mL. Sawant et al. (2007) determined the antimicrobial susceptibility of enteric bacteria isolated from the faeces of healthy lactating cattle. The MIC_{50} for 223 *E. coli* isolates was 4 µg/mL. EUCAST (2017) data indicated a MIC_{50} of 2 µg/mL for *E. coli*.

Table 7 summarizes the MIC_{50} values of intestinal microbiota for ampicillin.

A MIC_{50} can be derived for the wild-type distribution of *E. coli* and *Enterococcus* spp.

Several studies were available on the effects of orally administered ampicillin on the intestinal microbiota of humans and experimental animals. Oral treatment of mice with ampicillin significantly decreased endogenous intestinal

bacteria and colonization resistance at even the lowest dose of 114 µg/mL, which corresponds to a 1.2 g dose for a 70 kg human (Thijm & Van der Waaij, 1979). A no-effect level could not be determined for this study.

After six healthy volunteers who had not taken any antimicrobial drugs for at least 3 months received 1.5 mg of ampicillin or 15 mg of ampicillin plus 15 mg of streptomycin orally every day for 21 days, Corpet (1987) studied the impact on oral and intestinal microbiota. Ampicillin at 1.5 mg per dose showed no change over controls in total *E. coli* isolates in faecal samples, and the population of *E. coli* resistant to ampicillin showed individual variability with very little or no change in three of the five volunteers. Daily doses of 15 mg of ampicillin (plus 15 mg of streptomycin) showed a significant increase in the population of resistant *E. coli* isolates. Therefore, the no-effect concentration in humans was determined to be 1.5 mg per person. As the number of volunteers in the study was low, and the variability of the intestinal microbiota composition between and within individuals high (Cerniglia, Pineiro & Kotarski, 2016), the Committee applied a safety factor of 10 to derive an mADI of 0.25 mg/kg bw.

In a study using a germ-free mouse model associated with human faecal microbiota that was dosed with 0.5 or 8 µg/mL of ampicillin in drinking-water, the increase in resistant *E. coli* isolates, compared with control animals, persisted over the test period. However, the total population of faecal *E. coli* isolates did not change at either dose level. A no-effect concentration of ampicillin in mice was not determined because of insufficient data (Corpet, 1987).

After six healthy volunteers were administered 500 mg of ampicillin orally 4 times/day for 6 days, Midtvedt et al. (1986) evaluated seven microflora-associated characteristics (MACs) before and after the 6-day treatment and again 5 weeks after treatment: breakdown of mucin, formation of coprostanol, hydrolysis of bilirubin conjugates, formation of urobilinogen, formation of short-chain fatty acids, presence of β-aspartylglycine and inactivation of trypsin in faecal specimens. Ampicillin caused a reduction in short-chain fatty acids and urobilinogen after 6 days; at 5 weeks, levels were back to normal. The other MACs remained unaffected. The concentration of ampicillin was undetectable in faeces of 5/6 study participants; high β-lactamase activity was found in the five samples. A no-effect concentration could not be determined because this study did not test a range of doses.

Newton, Macfarlane & Macfarlane (2013) determined the effects of ampicillin (1.8 mg) on a consortium of 14 bacteria that included *Bacteroides*, *Bifidobacterium*, *Clostridium*, *E. coli*, *Enterococcus* and *Lactobacillus* species in chemostats operating at two dilution rates over a 48-hour incubation period. The bacterial species used in the chemostats represented different microbial communities occupying diverse nutritional niches within the intestinal microbiota. After ampicillin administration, culture dry weights, population

densities and short-chain fatty acids decreased for up to 24 hours at both dilution rates, but returned to original steady state levels after 48 hours. The effect of ampicillin on bacterial enzyme synthesis and activity depended on the dilution rate and the enzyme measured. A no-effect concentration could not be determined because dose-dependent studies were not conducted.

Step 2: Do residues enter the human colon?

Yes. Ampicillin studies were conducted to detect residues of ampicillin in the muscles of food fish species and food-producing animals. Muscle from fish tissue collected after withdrawal of medication contained relatively low amounts of ampicillin-derived residues (Son et al., 2011). Because even under the most conservative assumptions ampicillin residues may be present at low levels in meat and fish products, residues could enter the colon of a person ingesting these edible tissues.

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

Yes. Pharmacokinetic studies in humans indicate that ampicillin is rapidly absorbed after oral administration. In addition, ampicillin does not bind to human faecal suspensions and the faecal microbiota population was suppressed after ampicillin exposure.

Ampicillin is rapidly absorbed after oral treatment. The range of recovery in human urine is approximately 21–83%, depending on sampling time and fasting state. The dose does not influence the amount of ampicillin (in %) recovered in the urine. The variation between individuals is large (section 2.1.2(d)). Several studies gave different recovery results. The Committee decided to use the lowest mean value of ampicillin excreted in urine during fasting (21.0%, 0–4 h; Saito, Kato & Murakami, 1975) and non-fasting (21.8%, 0–8 h; Welling et al., 1977). The highest amount of a given dose recovered in urine was from fasting humans. Considering that intake of residues in food is not at fasting but from a meal, it is appropriate to consider the lowest recoveries found in urine. Numerous studies indicate that the parent drug ampicillin has microbiological activity. As there were no studies evaluating the microbiological activity of the metabolites (5*R*,6*R*)-ampicilloic acid, (5*S*,6*R*)-ampicilloic acid and ampicillin piperazine-2,5-dione, it was assumed that these ampicillin residues remain active in the human colon. In addition, studies by Hazenberg et al. (1984) with eight healthy humans indicated that ampicillin does not bind to faeces and that the intestinal microbiota from faecal specimens were highly susceptible to ampicillin. Midtvedt et al. (1986) also reported that faecal specimens of healthy human volunteers dosed with therapeutic levels of ampicillin have little or no detectable ampicillin and high β-lactamase activity.

The lowest ampicillin urinary recovery data from the human studies was 21.0%. Therefore, the fraction of oral dose available is conservatively estimated to be 79%.

Step 4: Is there any scientific justification to eliminate testing for either one or both end-points of concern?

Yes, there is potential for adverse effects in human intestinal microbiota with disruption of the colonization barrier because ampicillin and its metabolites do occur in human and experimental animals in small amounts. The susceptibility to ampicillin of a wide range of Gram-negative and Gram-positive microorganisms representative of those in the gastrointestinal tract suggests that ampicillin could disrupt the barrier effect that prevents colonization of pathogenic bacteria in the gut.

Ampicillin is a broad-spectrum β-lactam antibiotic used in both human and veterinary medicine (EFSA, 2012). The widespread use of this antibiotic at therapeutic doses has given rise to resistant bacterial strains and could reduce its efficacy in the treatment of disease. Less is known about exposure to residue levels of ampicillin in the gastrointestinal tract and the selection and development of resistance of the representative human intestinal microbiota (Wlodarska et al., 2011). Therefore, the Committee recommended that in vitro or in vivo studies be conducted using a range of ampicillin concentrations residual to therapeutic levels to determine no-observed-adverse-effect concentrations for increase in the population of resistant bacteria, according to VICH GL36(R) (2012).

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

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

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

where:

- MIC_{calc} for colonization barrier disruption 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). MIC_{calc} values for several bacterial species are shown in Table 8. The MIC_{calc} is derived as follows:

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

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 content:* The Committee adopted the 500 mL value, based on the volume measured in humans (Pritchard et al., 2014), and used it instead of 220 g in the evaluation.
- *Fraction of an 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. 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.

  The fraction of oral dose available to microorganisms was 0.79, a value conservatively estimated based on studies in humans in which approximately 21% of ampicillin and metabolites were recovered in urine. Therefore, the fraction of oral dose available to microorganisms in the colon is 1–21% = 79%.
- *Body weight:* An adult human is assumed to weigh 60 kg.

Therefore, the mADI for ampicillin is as follows:

$$\frac{0.387\ \mu g/mL \times 500\ mL}{0.79 \times 60\ kg\ bw} = 4.08\ mg/kg\ bw$$

For the effects on the colonization barrier, an mADI of 0–4 μg/kg was derived from in vitro MIC susceptibility testing data.

Based on the increase in the population(s) of ampicillin-resistant bacteria in the intestinal tract after exposure to a range of dose levels in humans, a NOAEL of 1.5 mg per person (equivalent to 0.025 mg/kg bw) was identified.

Table 8
Calculation of MIC_{calc}

Genera	MIC_{50} (µg/mL)	$Log_{2(MIC50)}$–$log_{2(0.12/2)}$
Bacteroides	16	8.06
Bifidobacterium	0.12	1
E. coli	2	5.06
Eubacterium	0.12	1
Fusobacterium	2	5.06
Peptostreptococcus	0.5	3.06
Clostridium	0.5	3.06
Lactobacillus	0.5	3.06
Enterococcus	1	4.06
Mean ($log_2(MIC_{50})$–$log_2(0.12/2)$)		3.71
Std dev ($log_2(MIC_{50})$–$log_2(0.12/2)$)		2.19
$t_{0.10, 8}$		1.397
Lower 90% confidence limit = 3.17–2.19 / √(9)*1.397		2.69
Back-transforming to the MIC scale = $2^{(2.69+log2(0.12/2))}$		0.387
MIC_{calc}		0.387

MIC_{50}: minimum concentration required to inhibit the growth of 50% of organisms; MIC_{calc}: 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); std dev: standard deviation; $t_{0.10,8}$: 90th percentile from a central *t*-distribution with degrees of freedom

The Committee derived an mADI of 0–2.5 µg/kg bw after rounding to 0–0.003 mg/kg bw and applying a safety factor of 10.

2.4 Observations in humans

2.4.1 Adverse effects

The most common adverse effects after therapeutic treatment with ampicillin at recommended oral doses of 17–33 mg/kg bw per day are diarrhoea and exanthema (occurring in ≥1% to <10% of cases). Less common adverse effects are nausea, vomiting, pseudomembranous colitis, itching, glossitis, stomatitis, urticaria, blood dyscrasias and mild allergic reactions (occurring in ≥0.1% to <1% of cases). Rare events include anaphylaxis and exfoliative dermatitis and erythema multiformae (occurring in ≥0.01% to <0.1% of cases; Swedish Medical Products Agency, 2018).

Ampicillin is contraindicated in patients known to be hypersensitive to penicillin. In rare cases, effects on liver (hepatitis and cholestasis icterus) and

kidney (interstitial nephritis and crystal urea) have been recorded (Pusey et al., 1983; Haddad, 1990; Castle, 2007). People with kidney disease may excrete ampicillin more slowly, thereby increasing levels in the body and increasing the risk for secondary effects.

In a prospective clinical study, 400 children were treated with ampicillin at daily doses of 50, 100, 150 or 200 mg/kg bw for at least 7 days. The incidence of erythematous maculopapular skin rashes was 3–7%, increasing with dose. A possible non-dose-related penicillin allergy was also observed in 3% of the treated children. Mild (18–30%) to moderate (2–11%) diarrhoea and mild vomiting (2–5%) were also common (Bass et al., 1973).

Maculopapular skin rashes are common adverse reactions to ampicillin; they are often not caused by an allergic mechanism but can be delayed hypersensitivity reactions to the side chain in ampicillin (Midtvedt, 1998).

2.4.2 Allergen and immune response

That ampicillin appears to have a lower allergenic potential than benzylpenicillin was shown in a study identifying maximum concentrations in skin-prick tests and intradermal testing of immediate allergic reactions. These maximum concentrations were 54 000 µmol/L for ampicillin and 0.05 µmol/L for benzylpenicilloyl, indicating that ampicillin has a significantly lower reactivity than benzylpenicilloyl (Torres et al., 2003). Radioallergosorbent tests (RAST) showed that the side chain of ampicillin was linked to a specific immunological reaction in some patients (Romano et al., 1997). However, no specificity of the response for ampicillin was found, as it showed a cross-reactivity with amoxicillin and other penicillins.

The risk for allergy was the main concern for residues of benzylpenicillin in food when benzylpenicillin and procaine penicillin were evaluated by the thirty-sixth JECFA (Annex 1, reference *91*). The benzylpenicilloyl metabolite was the main antigenic determinant. A few well-documented cases showed that hypersensitivity reactions could be a result of less than 40 µg of benzylpenicillin. The Committee at its fiftieth meeting (Annex 1, reference *134*) recommended that the daily intake of benzylpenicillin from food should be kept at less than 30 µg. This limit was also applied by the European Medicinal Agency (EMA) in 1992 when establishing MRLs (parent drugs as marker residues) for ampicillin and amoxicillin as well as for benzylpenicillin (Commission regulation 675/92/EU). Only the parent compounds was considered even if the penicilloic acid metabolite is responsible for the allergic reactions as it is bound to amino groups in tissues, milk and blood (EMA, 1996).

The literature search retrieved no data on hypersensitivity reactions caused by ampicillin residues in food. It is clear that there is cross-reactivity with

other β-lactam antibiotics. That ampicillin appears to have a lower allergenic potential than benzylpenicillin was shown by the maximum concentrations accepted in skin-prick tests and intradermal testing of immediate allergic reactions – 54 000 µmol/L for ampicillin versus 0.05 µmol/L for benzylpenicilloyl (Torres et al., 2003).

2.4.3 Gastrointestinal effects

In a systematic review of the use of antibiotics in otitis media in children, Coker et al. (2010) found that both ampicillin and amoxicillin caused lower rates of diarrhoea than, for example, cefixime (14% versus 21%).

Mild (18–30%) or moderate (2–11%) diarrhoea and mild vomiting (2–5%) were common and dose related, but severe diarrhoea was rare and not dose related in children treated orally with ampicillin at daily doses of 50, 100, 150 or 200 mg/kg bw for at least 7 days (Bass et al., 1973).

Of 56 patients treated with ampicillin at doses of 2–4.5 g, haemorrhagic colitis developed in eight (14.3%) of the patients (Sakurai et al., 1979).

2.4.4 Kidney toxicity

Like other penicillins, ampicillin is excreted through the kidney and therefore it may adversely affect the kidney. Rare cases of acute interstitial nephritis caused by hypersensitivity after repeated administration of ampicillin and amoxicillin have been reported (Pusey et al., 1983; Haddad, 1990). Neonates and infants should be administered the lowest possible dose as their kidneys are not yet fully developed. Although few articles on kidney toxicity in humans have been published, Nathanson et al. (2000) observed a permanent reduction of nephrons in pups in kidney development study in rats (section 2.2.5).

2.4.5 Reproductive toxicity

A population-based case–control study investigated if ampicillin given during the second and third months of pregnancy was teratogenic in humans (Czeizel et al., 2001). Data were from the Hungarian Congenital Abnormality Register (HCCSCA), covering a study population from the second trimester to the postnatal first year, in 1980 to 1996. The population controls were matched, with two newborn healthy infants for each newborn infant with congenital abnormalities (case group) according to sex, birth week and parents' residence. The oral dose of ampicillin was 250–500 mg 4 times/day for a mean of 5 days (range: 1–21) once or several times during pregnancy. The women included in the study were treated with only ampicillin or with ampicillin with other drugs. Of 38 151 pregnant women (representing 1.8% of all Hungarian births during the study period) that delivered babies without any malformations during the

study period, 2632 (6.9%) were treated with ampicillin alone. Of the 22 865 women with progeny with congenital abnormalities, 1643 (7.2%) were treated with ampicillin alone. Of all women treated with ampicillin alone (n = 4275), only 167 (3.9%) were treated with ampicillin alone. Risk for preterm delivery and prolonged pregnancy were lower in case patients than in the control group. Cleft palate with an odds ratio of 3.0 (95% confidence interval [CI]: 1.2–7.6) was the only abnormality with a significantly elevated odds ratio in the case–control pair analysis. The odds ratio for this abnormality was found if ampicillin use was higher during in the sensitive period for skeletal development, the second and third months of pregnancy. However, most women treated with ampicillin were also treated with other drugs. There was no difference of use of ampicillin during pregnancy between patient case group and the patient control group. The study authors noted that the observation of cleft palate was not consistent with that reported in other studies. The Committee considered that no firm conclusion could be drawn from the study.

The odds ratio for congenital cardiovascular malformations after use of ampicillin during pregnancy was 1.0 (Czeizel et al., 2001).

Several case–control studies found no evidence of cardiovascular effects when ampicillin was given during pregnancy (Jenkins et al., 2007).

A population-based case–control study assessed the incidence of neural tube defects in fetuses and liveborn infants in California, USA, in 1989–1991, in relation to medication use and maternal illness and fever. The odds ratios for ampicillin/amoxicillin was 0.81 (95% CI: 0.39–1.68). Ampicillin was not shown to increase neural tube defects when mothers were treated during the first trimester of pregnancy (Shaw et al., 1998).

3. Comments

3.1 Biochemical data

After oral administration, ampicillin was rapidly absorbed in mice, rats, dogs and humans, with a half-life in plasma of approximately 1 hour. The T_{max} was between 1 and 2.4 hours with C_{max} between 2.2 and 6.8 μg/mL at doses between 250 and 1000 mg per person (Knudsen, Rolinson & Stevens, 1961). Bioavailability of ampicillin in rats was 23% (English, Girard & Haskell, 1984) and in humans was 46% (Philipson, 1977). Plasma protein binding of ampicillin was only 17% in humans and cattle and 8% in horses (Acred et al., 1962; Brown, 1964). After oral treatment in humans and rats, ampicillin is metabolized to ampicillin piperazine-2,5-dione, (5*R*,6*R*)-ampicilloic acid and its epimer (5*S*,6*R*)-ampicilloic acid (Haginaka & Wakai, 1987; Haginaka et al., 1987).

There was a large variation in excretion of ampicillin in urine in humans; following oral treatment, mean excretion in different studies ranged between 21.8% and 42.8% in non-fasting subjects (Klein & Finland, 1963; Saito, Kato & Murakami, 1975; Welling et al., 1977; Eshelman & Spyker, 1978; Haginaka & Wakai, 1987). In the one study conducted with radiolabelled ^{35}S-ampicillin, radioactivity in faeces was 39 ± 19% at 7 days after a single dose of 500 mg per person (Swahn, 1975). Different foods ingested with ampicillin appear to affect excretion of ampicillin in urine (Saito, Kato & Murakami, 1975).

3.2 Toxicological data

Acute oral toxicity was tested in rats and mice. The oral LD_{50} was more than 5000 mg/kg bw in adult mice and rats (Acred et al., 1962), 3300 mg/kg bw for 1-day-old rats and 4500 mg/kg bw for 83-day-old rats (NTP, 1987).

In rabbits treated with ampicillin at doses of 5, 15 and 50 mg/kg bw for 3 days, there was more than 50% lethality at the lowest dose. In rabbits, many antibiotics cause gastrointestinal problems, like enteritis and disruption of the intestinal microbiota, that can cause death (Milhaud et al., 1976).

Mice were orally administered ampicillin trihydrate in corn oil at doses of 0, 200, 400, 800, 1600 or 2400 mg/kg bw per day of for 14 days (NTP, 1987). The NOAEL was 1600 mg/kg bw per day based on weight loss in males and diarrhoea at 2400 mg/kg bw per day (NTP, 1987).

Mice were administered ampicillin trihydrate in corn oil, by gavage, for 5 days a week for 13 weeks, at doses of 0, 250, 500, 1000, 2000 or 3000 mg/kg bw per day (representing 0, 179, 357, 714, 1430 and 2140 mg/kg bw per day for 7 days a week). No adverse effects were reported. The NOAEL was 2140 mg/kg bw, the highest dose tested (NTP, 1987).

Rats were administered ampicillin trihydrate in corn oil, by gavage, at doses of 0, 180, 370, 750, 1500 or 2 times 1500 mg/kg bw per day for 5 days a week (representing 0, 129, 264, 536, 1070 or 2140 mg/kg bw per day for 7 days a week) for 13 weeks. The NOAEL was 1070 mg/kg bw per day based on diarrhoea in the highest dose group (NTP, 1987).

Transgenic mice (Tg-*ras*H2) were orally treated, by gavage, with ampicillin trihydrate at 0, 350, 1000 or 3000 mg/kg bw per day for 26 weeks, while non-Tg mice were similarly treated with 3000 mg/kg bw per day. Tg-*ras*H2 mice treated with MNU as a single intraperitoneal dose of 75 mg/kg bw acted as positive control. Ampicillin trihydrate treatment did not affect mortality in either the Tg-*ras*H2 or non-Tg mice. Body weight was reduced in ampicillin trihydrate–treated male and female Tg-*ras*H2 mice from week 4 to 26 for the two lowest dose groups and week 5 to 26 for the highest dose group. Dilation of

caecum was observed in ampicillin trihydrate–treated non-Tg mice and dose-dependently in Tg-*ras*H2 mice. Several different tumours were found in the positive control group, demonstrating the carcinogenicity of MNU in Tg-*ras*H2 mice. Ampicillin trihydrate was not carcinogenic in Tg-*ras*H2 mice and non-Tg mice treated for 26 weeks. However, as decreased body weights and dilation of caecum were found in all treated groups, no NOAEL could be identified (Adachi et al., 2002).

Mice were administered ampicillin trihydrate in corn oil, via gavage, over 103 weeks at doses of 0, 1500 or 3000 mg/kg bw per day, 5 days a week (representing 0, 1070 and 2140 mg/kg bw per day for 7 days a week). Increased salivation and decreased activity were considered treatment related. Hyperplasia (granulocytic) of the bone marrow was observed in male mice (incidences of 16%, 28% and 28%, respectively), and hyperplasia in the mandibular lymph node in female mice (incidences of 3%, 19% and 24%, respectively). There was no treatment-related increase in neoplasms. In view of the hyperplasia in the treated groups, a NOAEL could not be identified.

Rats (F344/N) were treated, by gavage, with ampicillin trihydrate in corn oil for 103 weeks at doses of 0, 750 or 1500 mg/kg bw per day for 5 days a week (representing 0, 536 and 1070 mg/kg bw per day for 7 days a week). Clinical effects related to ampicillin trihydrate were diarrhoea, chromodacryorrhoea and excessive urination. Mononuclear cell leukaemia was observed in control and treated rats; however, this was not dose dependent. Mononuclear cell leukaemia was subsequently found to be a common spontaneous occurrence in aged rats of this strain. Focal cellular change of the adrenal cortex was increased in the treated rats (males: 1/50, 5/50 and 7/49, respectively; females: 6/50, 0/50, 12/50, respectively) but within historical control ranges (15/49). Non-malignant pheochromocytomas of the adrenal medulla were increased in male rats at the highest dose (13/50, 12/50 and 23/49, respectively; historical control data was 247/1092 [22.3%]). Hyperplasia in bone marrow was observed in male and female rats (12% [controls], 33% and 34% for males and 26% [controls], 45% and 50% for females, respectively). Hyperkeratosis was increased in males (6%, 14% and 20%, respectively) but not females; acanthosis in the forestomach was also increased in males (0, 4.5% and 11%, respectively) but not in females. In addition, inflammation in the prostate increased in high-dose male rats. No haematological, clinical chemistry and urine analyses findings were reported. A NOAEL could not be identified in this study because effects were seen at all doses (NTP, 1987).

Ampicillin was tested for genotoxicity in an adequate range of assays, both in vitro and in vivo. No evidence of genotoxicity was found (Jaju, Jaju & Ahuja, 1984; NTP, 1987; Stemp, Pascoe & Gatehouse, 1989; Watters et al., 2009; McNamee & Bellier, 2015; Magkoufopoulou et al., 2011).

The Committee concluded that ampicillin is unlikely to be genotoxic.

No reproductive toxicity studies in laboratory animals have been found.

In a developmental toxicity study, rats were treated orally with 0 and approximately 250 mg/kg bw per day of ampicillin trihydrate during gestation days 1–5, 6–11 or 12–17 (Solovev & Kovalenko, 1973). Fetal body weights were decreased on gestation days 6–11 and 12–17 and fetal cranio-caudal sizes were reduced on gestation days 12–17, measured at day 20 of embryonic development. No teratogenicity was observed. Effects were observed at the only dose tested.

No firm conclusion on reproductive or developmental toxicity could be reached from studies in experimental animals.

3.3 Observations in humans

A population-based case–control study investigated if ampicillin given during the second and third months of pregnancy was teratogenic in humans. Data were from the Hungarian Congenital Abnormality Register (HCCSCA), covering a study population from the second trimester to the postnatal first year, 1980–1996. The oral dose of ampicillin was 250–500 mg 4 times daily for a mean duration of 5 days (range: 1–21 days) once or several times during pregnancy. Of the 22 865 women with progeny with congenital abnormalities, only 1643 (7.2%) were treated with ampicillin. Cleft palate was the only abnormality with a significantly elevated odds ratio (3.0; 95% CI: 1.2–7.6) in the case–control pair analysis. Cleft palate was found after ampicillin use in the second and third months of pregnancy, the sensitive period for skeletal development, and the use of ampicillin was higher during pregnancy. The study authors noted that the observation of cleft palate was not consistent with reports from other studies (Czeizel et al., 2001). The Committee considered that no firm conclusion could be drawn from the study.

No evidence of cardiovascular effects was found in several case–control studies when ampicillin was given during pregnancy (Jenkins et al., 2007). In another case–control study, ampicillin was not shown to increase neural tube defects when mothers were treated for illnesses during the periconceptional period (Shaw et al., 1998).

The most common adverse effects in humans after therapeutic treatment (recommended doses 17–33 mg/kg bw per day) are diarrhoea and exanthema. In children treated orally with ampicillin at daily doses of 50–200 mg/kg bw for at least 7 days, mild to moderate diarrhoea and mild vomiting were common (Bass et al., 1973). Rare cases of acute interstitial nephritis after administration of ampicillin have been reported (Pusey et al., 1983; Haddad, 1990).

No data on hypersensitivity reactions caused by ampicillin residues in food were retrieved in the literature search. It is clear that there is cross-

reactivity with other β-lactam antibiotics. That ampicillin appears to have a lower allergenic potential than benzylpenicillin was shown in a study identifying maximum concentrations accepted in skin-prick tests and intradermal testing of immediate allergic reactions. The concentrations for ampicillin (54 000 μmol/L) and benzylpenicilloyl (0.05 μmol/L) indicated that ampicillin has a significant lower reactivity than benzylpenicilloyl (Torres et al., 2003).

3.4 Microbiological data

A decision-tree approach adopted by the sixty-sixth meeting of the Committee (Annex 1, reference *181*) that complies with VICH GL36(R) (VICH, 2012) was used by the Committee to determine the need for and to establish, if necessary, an mADI for ampicillin.

Ampicillin residues may be present at low levels in foods consumed by humans; therefore, ampicillin-related residues could enter the colon of a person ingesting edible tissues of treated food-producing animals. Ampicillin is rapidly absorbed after oral administration in humans and animals, and a considerable amount of the administered ampicillin was detected as unmetabolized parent compound in urine. Therefore, ampicillin residues entering the human colon will remain microbiologically active.

There is potential for adverse effects in the human intestinal microbiota with disruption of the colonization barrier because MIC values for the most relevant and predominant bacteria in the gastrointestinal tract indicate that they are susceptible to ampicillin. The Committee also found published reports on the increase in the population of ampicillin-resistant bacteria in the intestinal tract after exposure to residual to therapeutic concentrations that were used to determine no-observed-adverse-effect concentrations (NOAECs). Therefore, both microbiological end-points were evaluated to derive the mADI.

Studies of microbiological activity of ampicillin against bacterial strains representative of the human colonic microbiota were evaluated. Ampicillin was active against *E. coli* (MIC_{50} = 2 μg/mL), *Enterococcus* (MIC_{50} = 1 μg/mL), *Bifidobacterium* (MIC_{50} = 0.12 μg/mL), *Clostridium* (MIC_{50} = 0.5 μg/mL), *Bacteroides* (MIC_{50} = 16 μg/mL), *Lactobacillus* (MIC_{50} = 0.5 μg/mL), *Fusobacterium* (MIC_{50} = 2 μg/mL), *Eubacterium* (MIC_{50} = 0.12 μg/mL) and *Peptostreptococcus* (MIC_{50} = 0.5 μg/mL) (Sutter & Finegold, 1976; Appelbaum & Chatterton, 1978; Wexler et al., 1985; Louie et al., 1992; Burrows, Morton & Fales, 1993; Lim, Huh & Baek, 1993; Goldstein et al., 2004; D'Aimmo, Modesto & Biavati, 2007; Kheadr et al., 2007; Klare et al., 2007; EUCAST, 2017; Snydman et al., 2017).

The formula for deriving the mADI from MIC data is as follows:

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

where:

- MIC_{calc} for colonization barrier disruption represents the lower 90% confidence limit for the mean MIC_{50} for the most relevant and sensitive human colonic bacterial genera.

  The mADI was derived from in vitro MIC data, in accordance with Appendix C of VICH GL36 (VICH, 2004, 2012). The strains needed to determine the MIC_{calc} were chosen according to these guidelines, which state that an intrinsically resistant bacterial genus should not be included. Based on the genera with a MIC_{50} of *E. coli*, *Enterococcus*, *Bifidobacterium*, *Clostridium, Bacteroides*, *Lactobacillus*, *Fusobacterium*, *Eubacterium* and *Peptostreptococcus* (for individual MIC_{50} values, see above), the MIC_{calc} was calculated to be 0.387 µg/mL.
- *Mass of colon content:* The 500 mL value is based on the colon volume measured in humans.
- *Fraction of oral dose available to the microorganisms:* It is recommended that the fraction of an oral dose available for colonic microorganisms be based on in vivo measurements for the drug administered orally. Alternatively, if sufficient data are available, the fraction of the dose available for colonic microorganisms can be calculated as 1 minus the fraction (of an oral dose) excreted in urine. Human data are encouraged, but in their absence, 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 that of the parent compound. The fraction may be lowered if the applicant provides quantitative in vitro or in vivo data to show that the drug is inactivated during transit through the intestine.

  Ampicillin is rapidly absorbed and is excreted in urine primarily in unchanged form. The lowest ampicillin urinary recovery data from the human studies was 21%. Therefore, the fraction of oral dose available would be 1– 0.21 = 0.79.
- *The body weight* of an adult human is assumed to be 60 kg.

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

$$\text{ADI} = \frac{0.387\ \mu g/mL \times 500\ mL}{0.79 \times 60\ kg\ bw} = 4.08\ \mu g/kg\ bw$$

Therefore, an mADI of 0–0.004 mg/kg bw was derived from in vitro MIC susceptibility testing data.

Several studies on the effects of orally administered ampicillin on the intestinal microbiota of humans and experimental animals were available. Compared with control subjects, ampicillin administered at 1.5 mg doses to six healthy volunteers for 21 days showed no change in total *E. coli* isolates in faecal samples analysed by bacterial plate counts (Corpet, 1987). The population of *E. coli* resistant to ampicillin showed individual variability with very little or no change. Daily administration at 15 mg of ampicillin showed a significant increase in the population of resistant *E. coli* isolates. Therefore, the no-effect level in humans would be 1.5 mg/kg bw. Because the composition of the intestinal microbiota varies within and between individuals (Cerniglia, Pineiro & Kotarski, 2016), the Committee applied a factor of 10 to account for this variability, and derived an mADI of 0–0.0025 mg/kg bw.

The overall mADI for ampicillin was determined to be 0–0.0025 mg/kg bw based on studies on the increase in population of resistant bacteria in humans because the mADI value for this microbiological end-point is lower than that for microbiological effects on the disruption of the colonization barrier.

An acute reference dose (ARfD) was calculated as follows:

$$\frac{0.387\ \mu g/mL \times 3 \times 500\ mL}{0.79 \times 60\ kg\ bw} = 12.24\ \mu g/kg\ bw$$

Therefore, an ARfD based on microbiological effects of 0.012 mg/kg bw was derived from the disruption of the colonization barrier based on in vitro MIC susceptibility testing data.

4. Evaluation

Due to the absence of suitable data on ampicillin, it was not possible to establish a toxicological ADI. JECFA concluded that the most critical effect of penicillins used therapeutically is hypersensitivity (Annex 1, reference *208*). The limited

information available suggests that ampicillin is less chemically reactive than benzylpenicillin. The maximum tolerated concentrations of benzylpenicilloyl in skin-prick tests and intradermal testing was 0.05 μmol/L compared with 54 000 μmol/L for ampicillin, indicating that ampicillin has significantly lower allergenicity than benzylpenicillin (Torres et al., 2003).

Hypersensitivity, even if rare, is the most relevant toxicological effect of ampicillin and other β-lactam antibiotics in humans. However, no data on hypersensitivity reactions caused by ampicillin residues in food were retrieved in the literature search. The ampicilloyl metabolite of ampicillin was found to be the main antigenic determinant. Well-documented cases showed hypersensitivity reactions could be caused by ingestion of less 40 μg of benzylpenicillin. The Committee, at its fiftieth meeting, recommended that the daily intake of benzylpenicillin from food be kept at less than 30 μg (Annex 1, references *134*).

The microbiological end-points of disruption of colonization barrier and resistance were compared to derive the mADI.

For the effects on the colonization barrier, an mADI of 0–4 μg/kg bw was derived from in vitro MIC susceptibility testing data.

Based on the increase in the population(s) of ampicillin-resistant bacteria in the intestinal tract after exposure to a range of dose levels in humans, a NOAEL of 1.5 mg per person (equivalent to 0.025 mg/kg bw) was identified. The Committee established an mADI of 0–0.0025 mg/kg bw for ampicillin, rounded to 0–0.003 mg/kg bw, on the basis of this NOAEL of 0.025 mg/kg bw and using a safety factor of 10.

The Committee established an overall mADI of 0–0.003 mg/kg bw based on the increase in population of ampicillin-resistant bacteria in humans, and using a safety factor of 10. This microbiological end-point is lower than the mADI for effects on colonization barrier disruption.

In view of the large difference in sensitivity between benzylpenicillin and ampicillin, the Committee considered this ADI to be protective for potential allergenicity from residues of ampicillin.

The Committee established an ARfD of 0.012 mg/kg bw. The Committee considered that this ARfD would be protective for potential allergenicity from residues of ampicillin.

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Ethion (addendum)

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1. Explanation

Ethion (International Union of Pure and Applied Chemistry name: *O*,*O*,*O*′,*O*′-tetraethyl *S*,*S*′-methylene-*bis* (phosphorodithioate), Chemical Abstracts Service No. 563-12-2; Fig. 1) is an organophosphate pesticide for use mainly in agriculture and as a veterinary drug. In animals, ethion is used primarily as an acaricide to control ticks primarily in cattle as well as in sheep, goats, swine and horses.

Ethion was previously evaluated by Joint FAO/WHO Meeting on Pesticide Residues (JMPR) in 1968, 1972, 1986 and 1990 (FAO/WHO, 1969, 1972, 1987, 1991). Ethion is on the agenda for review by the Joint FAO/WHO Expert Committee on Food Additives (JECFA), using published data, at the request of the Twenty-third Session of the Codex Committee on Residues of Veterinary Drugs in Foods (FAO/WHO, 2016). The request was specifically in relation to setting maximum residue limits in edible tissues of cattle.

When used as a veterinary drug, ethion is indicated for the prevention of vector-borne diseases carried by the cattle tick *Rhipicephalus microplus* (syn: *Boophilus microplus*), as well as being used as an acaricide in sheep, goats, swine and horses. It is also used to control lice and flies.

For cattle, ethion has been formulated into immersion bath treatments, pour-on treatments, sprays and ear tags, often in combination with cypermethrin (a pyrethroid insecticide).

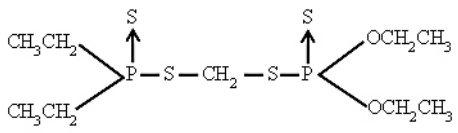
Immersion bath treatments are marketed in the form of a concentrate solution containing, for example, 40% ethion and 10% cypermethrin, which is then diluted with water to a suitable concentration (e.g. 400 parts per million ethion). The animals are then treated as a herd, by being corralled and driven through the bath one by one.

Pour-on products are marketed as solutions containing, for example, ethion at 150 g/L and cypermethrin at 50 g/L. Recommended doses are 5 mL for animals weighing 100–200 kg, 10 mL for animals weighing 200–400 kg and 20 mL for animals weighing more than 400 kg (range: 3.75–7.5 mg/kg body weight [bw] for all weights of animals).

Ear tags can contain 36–40 g ethion per ear tag. These are left on the animals for a specific time (e.g. 120 days). Some products recommend using one ear tag per animal; others recommend using two.

Withdrawal periods for the approved immersion bath formulations vary from 15–45 days for meat, depending on the specific formulation and jurisdiction. The ear-tag product reviewed has approved withdrawal periods of 0 days for meat and 2 days for milk.

Fig. 1
Structure of ethion



The primary mechanism of action of ethion, as with all organophosphate pesticides, is an irreversible inhibition of acetylcholinesterase (AChE), which degrades the neurotransmitter acetylcholine into choline and acetic acid. Acetylcholine is found in the central and peripheral nervous system, neuromuscular junctions and red blood cell (erythrocyte) membranes. The irreversible inhibition occurs in neural AChE in the peripheral and central nervous systems. In vitro studies showed that erythrocyte and neural AChE are inhibited with approximately the same concentrations of organophosphate compounds (Hayes, 1982). Erythrocyte AChE inhibition has been used as a peripheral surrogate marker for the activity of neural-acting AChE inhibitors. A plasma (or serum) cholinesterase, also known as plasma butyrylcholinesterase, is also inhibited by ethion. Studies in both humans and different animal species show that plasma cholinesterase is inhibited by ethion at exposure levels lower than necessary to inhibit neural and erythrocyte AChE. In humans and different animal species, toxic signs are generally not seen until at least 20% of erythrocyte AChE has been inhibited (Ecobichon, 1991).

Ethion is activated by cytochrome P450 isoforms to the corresponding oxon (mono- or di-) by oxidative desulfurization (Desouky, Abdel-Gawad & Hegazi, 2013). Ethion oxon then inactivates AChE by phosphorylating the serine hydroxyl group located at the active site of AChE. Once AChE has been inactivated, acetylcholine accumulates throughout the nervous system, resulting in overstimulation of the cholinergic system, producing uncoordinated muscle activity destroying its normal physiological control.

1.1 Comprehensive literature search

As part of the toxicological evaluation, the following bibliographical databases were searched using the term "ethion" and its synonyms[1] and Chemical Abstracts Service (CAS) number: Agricola (1976–August 2017), CAB Abstracts (1958–August 2017), Cochrane Library (2000–August 2017), Embase (1963–August 2017), FSTA (1969–August 2017), International Pharmaceutical Abstracts

[1] Synonyms: phosphorodithioic acid, *S*,*S*′-methylene *O*,*O*,*O*′,*O*′-tetraethyl ester; diethion; ethopaz; ethyl methylene phosphorodithioate ([(EtO)2P(S)S]2CH2); ENT 24,105; Fosfatox E; Hylemax; Hylemox; Niagara 1240; Nialate; *O*,*O*,*O*′,*O*′-tetraethyl *S*,*S*′-methylene bisphosphorodithioate; Phosphotox E; Rhodocide; Rodocid; Rodocide; RP 8167; AC 3422; *bis*(*S*-(diethoxyphosphinothioyl)mercapto)methane; Embathion; Ethanox; Ethiol; Ethodan; FMC-1240; Fosfono 50; Itopaz; Kwit; methyleen-*S*,*S*′-*bis*(*O*,*O*-diethyl-dithiofosfaat); *S*,*S*′-methylen-*bis*(*O*,*O*-diaethyl-dithiophosphat); methylene-*S*,*S*′-*bis*(*O*,*O*-diaethyl-dithiophosphat); *S*,*S*′-methylene *O*,*O*,*O*′,*O*′-tetraethyl phosphorodithioate; NA 2783; NIA 1240; phosphorodithioic acid, *O*,*O*-diethyl ester, *S*,*S*-diester with methanedithiol; *O*,*O*,*O*′,*O*′-tetraaethyl-*bis*(dithiophosphat); *O*,*O*,*O*′,*O*′-tetraethyl *S*,*S*′-methylenebisphosphordithioate; tetraethyl *S*,*S*′-methylene *bis*(phosphorothiolothionate); *O*,*O*,*O*′,*O*′-tetraethyl-*S*,*S*′-methylene di(phosphorodithioate); RP-Thion; Vegfru-fosmite; Ethiol 100.

Table 1
Inclusion and exclusion criteria for the comprehensive literature search

Inclusion criteria	Exclusion criteria
Any article on: • ethion concentrations in plasma of cattle or other ruminants • ethion concentrations in edible tissues of cattle or other ruminants • ethion residue determination methods for cattle plasma/tissue • ethion metabolism / metabolites in cattle • bioavailability of ethion residues in animals Articles in all languages	Any article focusing on: • ethion efficacy against target parasites • parasite resistance to ethion • ethion use in food animal species other than ruminants • kinetics/residues of organophosphates other than ethion (and not including ethion for comparison) • pharmacokinetics or pharmacodynamics of ethion in parasite species

(1970–August 2017), Global Health (1966–August 2017) and PubMed (1966–August 2017). In total 2569 articles were recovered, of which 738 were duplicates and were removed. The 1831 remaining titles and abstracts were scanned for information on pharmacodynamics, pharmacokinetics, short- and long-term toxicity, genotoxicity, reproductive and developmental toxicity, endocrine disruption or carcinogenicity of ethion in humans, laboratory animals (mice, rats, rabbits), chickens, dogs, goats, sheep, pigs and cattle.

As part of the evaluation of ethion residues, the Committee performed a comprehensive literature search in April 2017 using the PubMed, B-on, Springer Nature, Science Direct and Web of Science online databases to identify other relevant information.

The criteria applied to filter the articles for the assessment by the Committee at the present meeting are shown in Table 1.

Although no time limits were placed on the search results, studies published after 1994 were evaluated more thoroughly as these were not evaluated by the previous JMPR review of ethion (FAO/WHO, 1995).

The literature search retrieved no data useful in filling the identified gaps. The majority of papers determined to be relevant for establishing the maximum residue limit of ethion in cattle concentrated on specific analytical methodologies, usually for use in national or regional surveillance and control of residues of pesticides in foods (including cattle tissues and milk), and these were usually multi-residue methods. All the methods described analysed for parent ethion only.

There was some potentially useful information on the stability of ethion in various matrices during storage of samples before analysis.

There were no papers evaluating the pharmacokinetics or residues depletion of ethion or ethion metabolites in cattle.

The present report and monograph addendum were based on the summary JMPR evaluations from 1968, 1972, 1986 and 1990 (FAO/WHO, 1969, 1972, 1987, 1991); a United States Environmental Protection Agency (USEPA) report prepared in 1999; and a 2000 report and 2011 addendum from the Agency for Toxic Substances and Disease Registry (ATSDR). The Committee considered evaluations of studies on short and long-term toxicity, reproductive and developmental toxicity, genotoxicity and carcinogenicity and delayed neurotoxicity and on observations in humans.

2. Biological data

2.1 Biochemical aspects

2.1.1 Absorption, distribution and excretion

In a study of unspecified good laboratory practice (GLP) status, Selim (1985a) administered oral doses of [^{14}C]ethion (98.4% pure, labelled in the methylene position) to Sprague Dawley rats (5/sex). Both male and female rats received low single doses of ethion at 2 mg/kg bw, or high single doses of 105 mg/kg bw (males) 10 mg/kg bw (females). Another group (5/sex) received oral doses of non-radiolabelled ethion at 2 mg/kg bw per day for 14 days and a single dose of radiolabelled ethion at 2 mg/kg bw on day 15.

Seven days after the low single or low multiple doses, 93–94% of the administered radioactivity – 85% in urine and 4–7% in faeces – had been excreted by both male and females; within 3 days, 3–4% had been excreted in expired air. Similar values were observed for the female rats administered the high single dose (10 mg/kg bw). In the male rats administered the high single dose, 75% of the administered radioactivity had been excreted in urine and 15% in faeces 7 days after administration; after 3 days, 4% had been excreted in expired air.

Multiple pretreatments of both male and female rats with unlabelled ethion slightly increased the percentage of radioactivity excreted in the urine in the first 24 hours: 71% excreted by the males and 65% by the females without pretreatment; and 78% excreted by the males and 75% by the females after pretreatment with unlabelled ethion.

Levels of ^{14}C-radiolabelled residues were detected in all tissues of all animals in all treatment groups. Total radioactivity recovered from the 20 tissues examined 3 days after [^{14}C]ethion was administered was 0.36% in the high-dose males and 0.26–0.29% in all other groups. The highest concentrations of radioactivity were found in liver (0.1–7.0 mg/kg), kidneys (0.04–3.9 mg/kg),

adipose tissue (0.02–7.5 mg/kg) and, markedly on occasion, in hair (0.02–11.2 mg/kg) of both male and female rats.

In a related study, Selim (1985b) measured radioactivity in blood in Sprague Dawley rats (5/sex) fasted for 18 hours and then given an oral dose of approximately 0.38 mg/kg bw of [^{14}C]ethion (98.4% pure, labelled in the methylene position, in corn oil).

Peak blood levels of radioactivity were reached after about 6 hours in both male and female rats, indicating that ethion was slowly absorbed. Radioactivity levels in the blood of all animals were detected 72 hours after dosing, indicating a slow decline.

2.1.2 Biotransformation

In a toxicokinetic study (unspecified GLP status), Selim (1985c) analysed the urine of Sprague Dawley rats (5/sex) after single or multiple doses of [^{14}C]ethion (see section 2.1.1 for study outline) by solvent partitioning with ethyl acetate and subsequent high-performance liquid chromatography (HPLC) of the aqueous and organic phases.

Analysis indicated the presence of four to six distinct radioactive products. While HPLC analysis indicated the per cent distribution of radioactivity was the same in high-dose male and female rats as in low-dose male rats, a different per cent distribution was observed in the urine of both male and female rats after multiple doses as well as in low-dose female rats. In all cases, after solvent partitioning of urine, over 99% of the radioactivity was found in the aqueous phase, suggesting the presence of only nonsignificant levels (<1%) of organosoluble ethion or metabolites, such as ethion monoxon or ethion dioxon, in the urine. In addition, HPLC analysis of aqueous and organic phases after acidification showed no radioactivity in the fractions known to correspond with these three compounds.

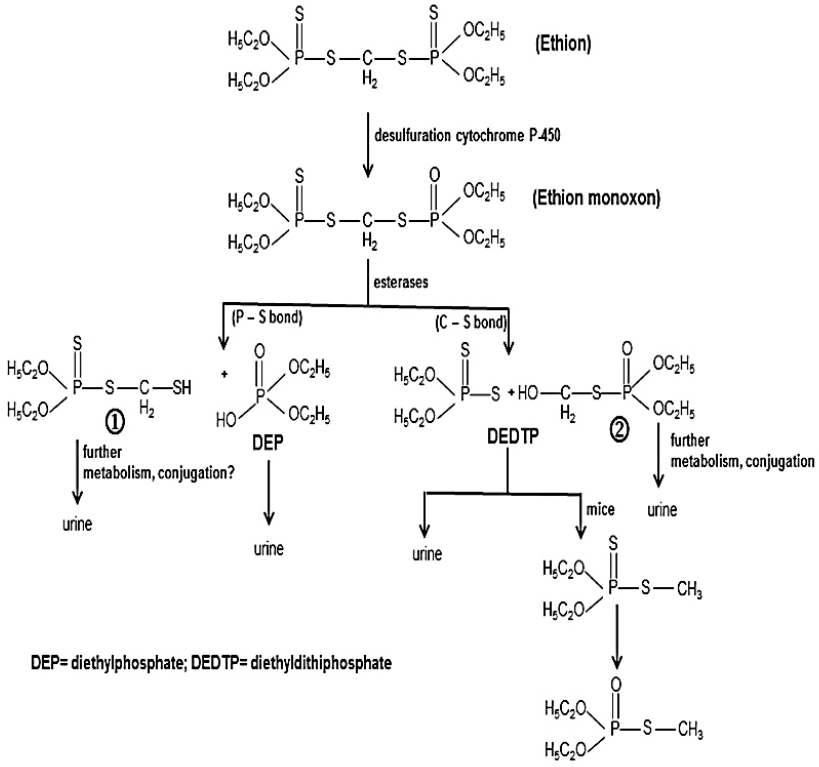
In conclusion, following absorption after administration of the oral dose of [^{14}C]ethion to male and female rats, the compound readily metabolizes into four to six polar, water-soluble products, which are excreted in the urine; however, none migrated with standards for ethion, ethion monoxon or ethion dioxon, and none were identified (Selim, 1985c).

Pathways for the biotransformation of ethion (Fig. 2) were proposed based on the studies of Rao & McKinley (1969), Nigg, Stamper & Mallory (1993) and Mahajna, Quistad & Casida (1996).

2.1.3 Effects on enzymes and other biochemical parameters

A GLP-compliant in vitro study examined the effect of ethion (200 µg/mL) on erythrocytes of blood of adult male rats. Treatment resulted in decreases in

Fig. 2
Proposed mammalian pathways of ethion biotransformation [a]



[a] Based on studies by Rao & McKinley (1969); Nigg, Stamper & Mallory (1993); and Mahajna, Quistad & Casida (1996)
Source: ATSDR (2000)

glucose-6-phosphate dehydrogenase (–27.29%), glutathione content (–4.42%), Na^+/K^+-ATPase (–25.00%), Ca^{2+}-ATPase (–36.50%) and AChE (–90.55%) and increases in the activity of glutathione reductase (+22.40%) and glutathione-*S*-transferase (3.92%) (Singh, Sandhir & Kiran, 2006).

2.2 Toxicological studies

Industrial Bio-Test Laboratories (IBT Labs) generated a substantial amount of data from toxicological studies with ethion, but were subsequently found to have engaged in scientific misconduct. In 1983, the IBT Review Program, led jointly by the USEPA and Health and Welfare Canada (now Health Canada), was initiated

Table 2
Summary of acute toxicity studies with ethion

Species	Route	LD_{50} (mg/kg bw), sex	Reference
Mouse	Oral	69, sex not specified	May and Baker, Ltd (1960)
Rat	Oral	97, M	Hazleton Laboratories, Inc. (1961)
Rat	Oral	65, M	Gaines (1969)
		27, F	
Mouse	Subcutaneous	630, sex not specified	May and Baker, Ltd (1960)
Rat	Subcutaneous	380, M	May and Baker, Ltd (1960)
		360, F	
Rat	Intraperitoneal	100, M, weanling	Brodeur & DuBois (1963)
		128, M, adult	
Rat	Dermal	245, M	Gaines (1969)
		62, F	

bw: body weight; F: female; LD_{50}: median lethal dose; M: male

to re-examine the reported toxicological effects of many pesticides, including ethion. As a result, some studies were rejected and others validated. Only those IBT Labs–generated studies of ethion that were reviewed and validated were included in the Committee's deliberations.

Overall, the Committee considered that the database was adequate to assess the risks of ethion.

2.2.1 Acute toxicity

The results of non-GLP-compliant oral (mice and rats), subcutaneous (rats) and dermal (rats) acute toxicity studies with ethion are summarized in Table 2.

No clinical signs, haematological examinations or histopathological data were included in the May and Baker Ltd (1960) and Hazleton Laboratories, Inc. (1961) acute studies cited in the 1968 JMPR (FAO/WHO, 1969).

In a study comparing the acute toxicity of ethion in weanling and adult rats (strain not reported), the median lethal dose (LD_{50}) for ethion (dissolved in 20% ethanol and 80% propylene glycol) administered intraperitoneally was 100 mg/kg bw in weanling rats and 128 mg/kg bw in adult rats. No clinical signs or haematological or histopathological evaluations were reported (Brodeur & DuBois, 1963).

In a non-GLP-compliant study, Gaines (1969) investigated the acute oral and dermal toxicity of ethion in Sherman rats (60 females, 80 males). Ethion was administered by gavage, using peanut oil as the solvent, at 5 mL/kg bw. Oral LD_{50} values were 65 mg/kg bw for males and 27 mg/kg bw for females. The minimum survival time was 3 hours for males and 4 hours for females; the maximum survival

time was 9 days for males and 6 days for females, with the lowest kill dose at 50 mg/kg for males and 20 mg/kg bw for females. No clinical signs, haematological or histopathological evaluations were reported.

In the dermal part of the study, ethion (purity not stated) was dissolved in xylene and applied at a rate of 1.6 mL/kg bw onto clipped skin on the shoulder and back of rats (about 3.0 × 4.5 cm). The clipped area of unbroken skin was bathed with a 1:1 solution of acetone and 95% ethyl alcohol. The rats were exposed to ethion without any restraint or covering of the treated area, which could have led to oral or respiratory exposure.

The dermal LD_{50} was 245 mg/kg bw in males and 62 mg/kg bw in females. The minimum survival time was 3 hours for males and 6 hours for females, and the maximum survival time was 7 days for males and 3 days for females, with the lowest kill dose at 150 mg/kg for males and 50 mg/kg for females. No clinical signs, haematological or histopathological evaluations were reported (Gaines, 1969).

In a GLP-compliant study, young Wistar rats (n = 6 females) underwent a 6-hour feed-and-fast cycle before being weighed and administered, by gavage, ethion (purity: 95.4%) dissolved in saline solution. A progression according to the Organisation for Economic Co-operation and Development Guideline 425 (up-and-down procedure) was followed, starting with a dose of 175 mg/kg bw, and alternating with a dose of 550 mg/kg bw, until three animals were treated with each dose. The animals were observed hourly for the first 6 hours, then at 12, 24, 36 and 48 hours and daily until day 14 after administration of the substance.

Clinical manifestations (e.g. exophthalmia, ptialism, presence of urine in the vagina, which was a purple colour) and tonic–clonic convulsions were observed in all animals treated at 550 mg/kg bw up to 4 hours after administration. One animal was found dead at 4 hours post administration, and two others in this group were euthanized 12 hours after dosing. No symptoms were observed in females at 175 mg/kg bw. Findings at necropsy included intestinal hyperaemia and slightly paler colour of liver in animals from both groups. No treatment-related histopathological effects were observed in any organ in rats from either group. Analysis using software AOT425StatPgm determined an LD_{50} of 310 mg/kg bw, with a 95% confidence interval of between 175 and 550 mg/kg bw (Ortega, 2017).

2.2.2 Short-term studies of toxicity

(a) Oral administration

(i) Rats

Data from non-GLP-compliant short-term toxicity studies in rats evaluated by JMPR in 1968 and ATSDR in 2000 are presented.

In a 13-week non-GLP-compliant study, male and female rats were fed diets containing ethion at concentrations of 0, 3, 10, 30 or 100 mg/kg feed (equal to 0, 0.15, 0.5, 1.5 and 5.0 mg/kg bw per day). Significant inhibition of erythrocyte cholinesterase activity was observed at concentrations above 0.15 mg/kg bw per day. All animals exhibited normal physical appearance, behaviour, growth and feed consumption. No histopathological changes were noted (Keller & Paynter, 1958).

In a 13-week non-GLP-compliant study, male and female rats received diets containing ethion at concentrations of 0, 300, 600, 1000 or 1500 mg/kg feed (equal to 0, 15, 30, 50 and 75 mg/kg bw per day). Complete inhibition of erythrocyte cholinesterase activity occurred in all test groups, except males at 15 mg/kg bw per day, where inhibition was marked but not complete. No gross or histopathological treatment-related changes were observed in the surviving rats (Keller & Paynter, 1958).

Based on erythrocyte AChE inhibition in both rat studies, a no-observed-adverse-effect level (NOAEL) of 0.15 mg/kg bw per day was identified.

In two distinct rat studies, the body weights and histopathology of different organs were evaluated. In one experiment, male and female albino rats (strain not reported) received 10 mg/kg of ethion (equal to 0.5 mg/kg bw) in their feed for 96 days (Keller & Paynter, 1958). In the other study, Sprague Dawley rats (80/sex per group) received 0, 0.1, 0.2 or 2 mg/kg bw of ethion for 6, 12 or 18 months (Morrow & Mayhew, 1985). Results showed that there were no treatment-related effects on body-weight gain or histopathology of the respiratory tract, liver, kidney and bladder, heart, aorta, gastrointestinal tract, pituitary, thyroid, parathyroid, thymus, adrenals, skin and eye.

In a GLP-compliant study, Bhatti, Kiran & Sanhir (2010) administered ethion by gavage to adult male Wistar rats at 0 or 2.7 mg/kg bw per day for up to 28 days. The rats were killed after 7, 14, 21 or 28 days. A significant decrease in body weight was observed in treated rats throughout treatment. Histopathological examinations showed severe mononuclear infiltration in all portal areas, severe hydropic degeneration and focal lobular hepatitis in the ethion-treated group.

(ii) Dogs

Purebred beagle dogs (4/sex per dose) were administered ethion (purity: 93.4%) in the diet at dose levels of 0, 0.5, 2.5, 25 or 300 mg/kg feed for 90 days (equivalent to approximately 0, 0.01, 0.06, 0.71 and 6.9 mg/kg bw per day for males and 0, 0.012, 0.07, 0.71 and 8.25 mg/kg bw per day for females, respectively). The dogs were examined twice a day for mortality and morbidity and once a day for clinical signs of toxicity. Body weight and feed consumption were determined weekly.

Clinical pathology parameters, which included cholinesterase activities (plasma, erythrocyte, brain), haematology and clinical chemistry, were evaluated

in all dogs prior to treatment initiation (day 16) and during weeks 5, 9 and 13. All surviving animals were killed and histopathological examinations of the following tissues were conducted: lesions, brain (with medulla/pons, cerebellar cortex and cerebral cortex), gallbladder, pituitary, thyroid (parathyroid), thymus, lungs, trachea, heart, bone (femur), salivary glands (mandibular), bone marrow (sternum), kidneys, uterus, adrenals, liver, spleen, pancreas, testes (with epididymides), ovaries, aorta, oesophagus, stomach, duodenum, jejunum, ileum, colon, caecum, rectum, urinary bladder, mesenteric lymph node, sciatic nerve, spinal cord (cervical, thoracic, lumbar), skin, mammary glands and eyes.

One female at 300 mg/kg feed was euthanized on day 90 (1 day before scheduled kill) after exhibiting clinical signs of emesis, dehydration and reduced body mass. All other animals survived until the scheduled kill, although the animals in the highest dose group were generally in poor condition by study end, with clinical signs including miosis (all animals), emesis (all animals), dehydration (3 males, 2 females), salivation (2 males, 2 females) and tremors (3 males, 4 females). Erythrocyte AChE was inhibited at weeks 5 (94% males, 96% females), 9 (95% males, 93% females) and 13 (94% males, 93% females) in the highest dose groups only. At termination, brain AChE activity in animals at 300 mg/kg feed was inhibited by 64% in males and 61% in females; in animals at 25 mg/kg feed, brain AChE was inhibited 23% in males and not in females. Plasma cholinesterase inhibition was dose related in both males and females. No significant differences in absolute organ weights were observed between the treated and control animals, and no treatment-related histopathological effects were observed in any organ, including the brain, sciatic nerve and spinal cord (Bailey, 1988).

The NOAEL was 2.5 mg/kg feed (equivalent to 0.06 mg/kg bw per day) based on inhibition of brain AChE activity at 25 mg/kg feed (equivalent to 0.71 mg/kg bw per day).

(b) **Dermal application**

In a non-GLP-compliant study, rabbits (6/sex per dose) were dermally treated with technical grade ethion at doses of 0, 1, 3, 25 or 250 mg/kg bw for 21 days. An increased incidence of erythema and desquamation was observed at the application sites in both males and females at 25 and 250 mg/kg bw (Weiner, 1985).

(c) **Dermal sensitization**

In a non-GLP-compliant study, guinea-pigs (number and sex not specified) were dermally exposed to technical grade ethion in a skin sensitization test. Ethion

caused slight erythema, which cleared within 48 hours. Ethion was determined not to be a skin sensitizer (Freeman, 1984).

2.2.3 Long-term studies of toxicity and carcinogenicity

(a) Mice

In a non-GLP-compliant chronic toxicity and carcinogenicity study, male and female mice received ethion at 0, 0.75, 1.5 or 8 mg/kg feed (equal to 0, 0.11, 0.22 and 1.17 mg/kg bw per day for males and 0, 0.12, 0.24 and 1.28 mg/kg bw per day for females, respectively) for 105 weeks.

There were no treatment-attributable effects on body weight, feed consumption, incidence of mortality, haematology, blood biochemistry or erythrocyte or brain cholinesterase. Incidences of tumours were not influenced by treatment.

The NOAEL was 8 mg/kg feed (equal to 1.17 mg/kg bw per day), the highest dose tested (Morrow, 1985).

(b) Rats

Sprague Dawley CD albino rats (80/sex per group) were fed diets containing ethion technical (purity: 92.1%) at 0, 2, 4 or 40 mg/kg feed (equivalent to 0, 0.09, 0.18 and 1.8 mg/kg bw per day for males and 0, 0.11, 0.22 and 2.2 mg/kg bw per day for females, respectively) for 24 months. All animals were examined for mortality, morbidity, overt signs of toxicity, body weight and feed consumption throughout the study. Clinical blood and cholinesterase determinations were conducted on 10/sex randomly selected baseline animals and on 10 animals/sex per group prior to interim kills at 6, 12 and 18 months and the terminal kill at 24 months. All surviving animals underwent ophthalmological examination at 0, 12 and 24 months. Animals found dead, euthanized moribund or killed on schedule underwent gross necropsy and histopathological examination of about 30 tissues.

Survival at termination was 40–62% in males and 48–58% in females, significantly lower than in control animals at 12 (–37%) and 18 (–38%) months for males and at 6 (–49%), 12 (–55%), 18 (–57%) and 24 months (–46%) for females. Differences in mortality were not dose related and were considered not treatment related. There was a treatment-related decrease in serum cholinesterase in high-dose male and female rats. Haematological examination (red blood cell count, haematocrit, haemoglobin, total and differential white blood cell count, platelet count, mean corpuscular volume, mean corpuscular haemoglobin and mean corpuscular haemoglobulin concentration), blood biochemistry (aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, alkaline phosphatase, total bilirubin, glucose, urea nitrogen, total protein, albumin, globulin, cholesterol, Ca^{+}, Na^{+} and K^{+}), ophthalmological examination and

erythrocyte and brain cholinesterase activity measures indicated no treatment-attributable effect at any dose level. Compared with the control groups, there was an increase in body weight in low-dose males at 12 months and a decrease in testes weight in mid-dose males at 18 months; although statistically significant, these differences were neither consistent over the treatment period nor dose-dependent, and therefore were not considered treatment related.

Histopathological examination at interim and terminal kills indicated no neoplastic treatment-related changes. At termination, several non-neoplastic lesions in both male and female rats occurred at a higher incidence in treated animals than in controls. However, these non-neoplastic changes did not show a dose–response relationship and were considered within ranges encountered in long-term studies with Sprague Dawley rats. These changes, which were considered not treatment related, included C-cell hyperplasia of the thyroid in both sexes, vacuolation of the adrenal gland in males and uterine hydrometra in females (Morrow & Mayhew, 1985).

Based on the unchanged values of erythrocyte AChE in all dose groups, the NOAEL was 1.17 mg/kg bw per day, the highest dose tested.

2.2.4 Genotoxicity

(a) In vitro

Table 3 shows the in vitro studies performed to examine the genotoxic potential of ethion. ATSDR (2000) concluded that there was no evidence of genotoxicity in several in vitro studies. Ethion was negative in tests for point mutations (Kada, Moriya & Shirasu, 1974; Waters et al., 1980), DNA repair (Shirasu et al., 1976; Waters et al., 1980), recombination (Waters et al., 1980), sister chromatid exchange (Sobti, Krishan & Pfaffenberger, 1982) and unscheduled DNA synthesis (Waters et al., 1980).

(b) In vivo

In a non-GLP-compliant in vivo cytogenetics assay, the ability of ethion to induce numerical or structural chromosomal aberrations in rat bone marrow cells was investigated. Sprague Dawley rats (5 males/group) were either dosed with ethion (purity: 92.1%) in corn oil by oral gavage at 0, 4.7, 14, 47 or 140 mg/kg bw per day for 5 consecutive days or with a single intraperitoneal injection of 5 mg/kg bw triethylenemelamine (TEM) 1 day prior to scheduled kill (positive controls). All the animals at 140 mg/kg bw per day and one at 47 mg/kg bw per day died following administration of the second dose. Clinical signs of toxicity were reported in the animals in the two highest dose groups (47 and 140 mg/kg bw per day), but not in the animals receiving lower doses (4.7 or 14 mg/kg bw per day) or the control rats. Bone marrow cells collected from each surviving animal 6 hours

Table 3
Summary of in vitro genotoxicity studies with ethion

System	End-point	Results		References
		With activation	Without activation	
Prokaryotes				
Salmonella typhimurium TA98, TA100, TA1535, TA1537, TA1538	Point mutation	Negative	Negative	Haworth, Wagner & Coyle (1984)
S. typhimurium TA1535, TA1536, TA1537, TA1538	Point mutation	No data	Negative	Kada, Moriya & Shirasu (1974)
S. typhimurium TA100, TA1535, TA1537, TA1538	Point mutation	Negative	Negative	Waters et al. (1980)
Escherichia coli WP2*urvA*$^-$	Point mutation	Negative	Negative	Waters et al. (1980)
E. coli W3110, P3478	DNA repair	No data	All negative	Waters et al. (1980)
Bacillus subtilis H17, M45	DNA repair	No data	All negative	Waters et al. (1980)
B. subtilis H17, M45	DNA repair	No data	All negative	Shirasu et al. (1976)
Saccharomyces cerevisiae D3	Recombination	Recombination	Negative	Waters et al. (1980)
Eukaryotes				
Human lymphoid cells LAZ-007	Sister chromatid exchange	No data	Negative	Sobti, Krishan & Pfaffenberger (1982)
Human fetal lung fibroblasts WI-38	Unscheduled DNA synthesis	No data	Negative	Waters et al. (1980)

Source: ATSDR (2000)

after the final dose were arrested in metaphase, and examined for numerical or structural chromosomal aberrations.

The mitotic index (% of cells in mitosis) and the number of aberrations (gaps, breaks, fragments and rearrangements) were not significantly different in treated rats when compared with controls. Excluding gaps, the only aberration observed in treated rats was a chromatid break in one rat in the low-dose group. A significant increase in the number of chromosomal aberrations was noted in the positive control rats.

Under the conditions of the assay, ethion did not induce chromosomal aberrations in male rats (Putman & Melhorn, 1984).

In a non-GLP-compliant study, commercial ethion (Tafethion 50 EC) was examined for its ability to induce chromosomal aberrations and micronuclei (MN) in somatic (bone marrow) cells and sperm-shape abnormalities in germ cells of male and female Swiss albino mice tested in vivo (3/sex per group). The mutagenicity of commercial preparation of ethion was tested using three doses (15.0, 22.5 and 30.0 mg/kg bw), three routes of administration (intraperitoneal, oral and subcutaneous), three acute exposure times points (6, 24 and 48 hours) and chronic exposure (6 mg/kg per day; 5 days) for chromosomal aberrations. All three doses were also tested for micronucleus (at 30 hours) and sperm-shape abnormality (at 35 days).

Results showed that only those mice exposed intraperitoneally and acutely to ethion presented with an increased frequency of chromosomal aberrations in bone marrow. No alterations were detected in the micronucleus of bone marrow cells and sperm-shape abnormality was not detected in mice exposed to ethion (Bhunya & Behera, 1989).

In a non-GLP-compliant study, the genotoxicity of a commercial preparation of ethion (Tafethion) was evaluated in white Leghorn chicks by way of a chromosomal aberration assay. The chemical was tested using three different doses (20, 15 and 10 mg/kg bw), two routes of administration (intraperitoneal and oral) and three acute exposure time points (6, 24 and 48 hours) and chronic exposure (4 mg/kg bw per day; 5 days). An increase in the frequency of sister chromatid exchanges was observed in animals exposed to ethion (Bhunya & Jena, 1994).

2.2.5 Reproductive and developmental toxicity

(a) Multigeneration reproductive toxicity

In a non-GLP-compliant three-generation reproduction study (2 litters/generation), groups of 15 male and 30 female albino rats (Charles River CD strain) were designated as F_0 parental animals and given diets containing 0, 2, 4 or 25 mg/kg feed (equal to 0, 0.1, 0.19 and 1.2 mg/kg bw per day) of ethion technical (92.1% pure) in corn oil from day 30 until the end of the second lactation period (weaning of F_{1b} litters). To obtain the following generations, 15 males and 30 females from each treatment group were randomly selected from the second litters of each generation (F_{1b} and F_{2b} litters) and designated as F_1 and F_2 parental animals, respectively. Both F_1 and F_2 parental animals were 21–35 days old at the start of the F_1 and F_2 generations, respectively. All animals were observed for mortality, morbidity and overt signs of toxicity. All surviving parental animals underwent gross necropsy. Five rats/sex from the F_{1b}, F_{2b} and F_{3b} litters underwent gross pathology. Serum, erythrocyte and brain cholinesterase were determined at terminal kill of rats (10/sex per group) from the F_1 and F_2 parental generations. Pre-mating serum and erythrocyte cholinesterase was determined in 10 rats/sex per group.

The survival rate was good in all groups of the three generations. Small (<10%) but significant reductions in body weight were noted during the F_{2a} lactation period of F_1 females at 1.2 mg/kg bw per day. The study authors considered these reductions not treatment related. Occasional statistically significant differences in feed consumption between treated and control animals were observed; the study authors did not consider these attributable to ethion administration. No effects attributable to ethion administration were noted in antemortem observations; reproductive performance (including mating

index, male and female fertility indexes and gestation index); progeny survival (including mean numbers of pups delivered, delivered viable, stillborn, found partially cannibalized and surviving at lactation days 1, 4, 7, 14 and 21); or progeny body weight and development. A significant increase in erythrocyte cholinesterase inhibition was noted in F_1 males at 1.2 mg/kg bw at terminal kill compared with controls. There were no significant treatment-related decreases in serum, erythrocyte or brain cholinesterase in F_1 males or in erythrocyte or brain cholinesterase in F_1 females compared with controls. A significant reduction in serum cholinesterase (52.7%) was noted in F_2 females at 1.2 mg/kg bw at terminal kill compared with controls.

There were no significant differences between treated and control groups in erythrocyte or brain cholinesterase values for males or females of the F_2 generation. Gross pathological examination of F_0, F_1 and F_2 parental animals and F_{1b}, F_{2b} and F_{3b} progeny showed no alterations attributable to the administration of ethion at any dose level. There were no significant treatment-attributable effects in organ weights or organ-to-body-weight and organ-to-brain-weight ratios in parental or progeny animals at any dose or generation (Salamon, Enloe & Mayhew, 1985).

Based on the absence of changes in erythrocyte AChE levels, the NOAEL was 1.2 mg/kg bw per day, the highest dose tested.

(b) Developmental toxicity

In a non-GLP-compliant study, mated female rats (Charles River Crl:COBS CD (SD)BR strain; 25/group; 17 weeks old) were administered ethion technical (92.1% pure) in corn oil by gavage at doses of 0, 0.2, 0.6 or 2.5 mg/kg bw per day on gestation days 6 through 15 (based on observation of vaginal plug in situ or in the cage pan). The female rats were killed on gestation day 20 and, after caesarean section, examined for the number of pregnancies, implantations, early and late resorptions, live and dead fetuses and corpora lutea. Fetuses were weighed and sexed; approximately one third of each litter were examined for visceral variations and two thirds were examined for skeletal variations. Water consumption and uterine weights were not recorded in this study.

No rats died during the study. A treatment-related, statistically significant increase in the incidence of hyperactivity was found in the high-dose group. A slight but not toxicologically significant decrease in fetal body weight was observed in mid- and high-dose litters (0.9% and 2.1%, respectively). Slight, statistically nonsignificant increases in the incidence of fetuses with variations, of litters with fetuses with variations and of the per cent of fetuses per litter with one or more variations were observed in the high-dose group. Treatment-related skeletal variations consisted of delays in ossification of the ischium and pubes of

the pelvis in the mid- and high-dose fetuses and of the hyoid and manubrium in the high-dose fetuses; none of these variations occurred in any control or low-dose fetuses. Only the higher incidence of incomplete or absent ossification of the pubes observed in the high-dose group achieved statistical significance compared with controls ($P < 0.01$). The study authors considered these skeletal variations to be reversible developmental delays as a result of fetal toxicity at 0.6 and 2.5 mg/kg bw per day. Gross external, soft tissue and skeletal examination indicated no other treatment-attributable effects. There were no significant differences between treated and control groups in maternal body-weight gain or feed consumption, incidence of pregnancies, corpora lutea, implantations, live and dead fetuses, early and late resorptions or litter size, viability or sex ratio (Hoberman, Christian & Christian, 1983).

The NOAEL for maternal toxicity was 2.5 mg/kg bw per day, the highest dose tested.

The NOAEL for embryo/fetal toxicity was 0.2 mg/kg bw per day based on skeletal variations at 0.6 mg/kg bw per day.

2.2.6 Special studies

(a) Delayed neurotoxicity

In a non-GLP-compliant study, the acute delayed neurotoxicity of ethion was assessed in adult domestic hens (10/dose). The study consisted of two parts: in the first part, the LD_{50} was determined using gavage doses of ethion of 0, 889, 1333, 2000, 3000 and 4500 mg/kg bw. The LD_{50} was 2792 mg/kg bw (14-day observation period). Most deaths occurred 1–48 hours after dosing. Toxic signs included unsteadiness, wing-dropping, inability to stand, trembling and reductions in body weight. No effects were seen at 889 mg/kg bw.

In the second part of the study, neurotoxicity was assessed in groups of 10 hens. A vehicle control group was dosed orally with corn oil, a positive control group with 500 mg/kg tri-*o*-cresyl phosphate (TOCP) and each of four test groups with ethion at the LD_{50} (2792 mg/kg bw). The birds dosed with ethion were protected with 10 mg/kg bw intramuscular atropine sulfate. Dosing was followed by a 21-day observation period, a re-dosing and a final 21-day observation period. Surviving hens were killed at 42 days. Histopathological examination of brain, spinal cord and peripheral nerve tissues was performed on 25/40 animals dosed with ethion that had survived for 42 days, 10/10 control hens and 10/10 TOCP-treated hens.

Clinical signs of toxicity included unsteadiness, leg stiffness, stumbling, inability to stand and reduced feed consumption and body weight in the 3 days following each of the two administered doses. However, no unusual clinical signs of neurotoxicity, gross pathological organ changes or neuropathological (i.e. histological) effects on the control or peripheral nervous system were observed.

In contrast, hens treated with TOCP displayed ataxia as well as significant axonal degeneration in the brain and spinal cord and peripheral nerves (Roberts et al., 1986).

2.3 Microbiological effects

Considering the chemical structure and the mode of action of ethion, the Committee did not anticipate any adverse effects of ethion residues on human gastrointestinal microbiota.

2.4 Observations in humans

Ethion was administered to a group of six male volunteers at levels of 0, 0.05, 0.075, 0.1 and 0.15 mg/kg bw per day for a period of 3 weeks at each dose level, with the exception of the highest dose group (0.15 mg/kg), who were administered ethion for 3 days only. The control group consisted of four individuals. The ethion was administered in divided doses in gelatine capsules as a solution in corn oil 3 times a day. Plasma and erythrocyte cholinesterase activities were determined 5 times during 2 weeks of the pretest period and 3 weeks of the test period.

No significant depression in erythrocyte cholinesterase was noted. A significant depression in plasma cholinesterase of 21% and 29% was noted for dose levels of 0.1 and 0.15 mg/kg per day, respectively. Plasma cholinesterase activity at the highest dose level returned to pretest values in 7 days. The 0.075 mg/kg per day level was considered borderline, with the mean inhibition at 15%. No adverse effects were noted in any of the other parameters examined, including haemoglobin concentration, haematocrit, red blood cell count, total differential leukocyte counts, blood pressure, pulse rate, pupil size, light reflex, eye accommodation, chest sound, muscle tone, knee-jerk, and tongue and finger tremor.

Based on the absence of effects on erythrocyte AChE activity at any dose tested, a NOAEL of 0.15 mg/kg bw was derived, the highest dose tested (Palazzolo, Fancher & Calandra, 1970).

3. Comments

3.1 Biochemical data

Ethion is a small (molecular weight 384 g/mol) lipid-soluble molecule that can be absorbed by passive diffusion through the lungs, gastrointestinal tract and skin. A study in rats (Selim, 1985b) found oral absorption of ethion to be rapid.

Gastrointestinal absorption of ethion appears to be 80% in the rat, based on residue studies performed with [^{14}C]ethion (Selim 1985a). In this study, 75–85% of the total label was excreted in urine and 4–8% in faeces, irrespective of whether labelled ethion was administered as the final dose after 14 days of dosing with unlabelled ethion.

Although general characteristics of organophosphate metabolism are known, most ethion metabolites have not been identified. Ethion is converted via oxidative desulfuration by hepatic cytochrome P450 enzymes to its active oxygen analogue, ethion monoxon (Rao & McKinley, 1969). Ethion monoxon is a highly reactive and potent inhibitor of cholinesterases; it reacts with and inhibits neural AChE. It is not known if ethion monoxon can be further desulfurated to ethion dioxon. Ethion and ethion monoxon are further metabolized by blood and liver esterases. Cleavage of the monoxon at the P–S bond results in diethyl phosphate and a transient intermediate (*O*,*O*-diethyl-*S*-mercaptomethyldithiophosphate). In humans, cleavage can occur at both the P–S bond and the S–C bond, based on the detection of diethyl phosphate (P–S cleavage of the monoxon), diethyl thiophosphate (P–S cleavage of ethion or S–C cleavage of the monoxon) and diethyl dithiophosphate (S–C cleavage of ethion) in the urine of pest control workers who use ethion; however, relative amounts of the identified substances were not reported (Nigg, Stamper & Mallory, 1993). Evidence in mice of cleavage at the S–C bond and subsequent methylation of sulfur has also been reported (Mahajna, Quistad & Casida, 1996).

Elimination of ethion is mainly via water-soluble metabolites in the urine. Analysis of urine of rats dosed with [^{14}C]ethion, using HPLC, found four to six unidentified metabolites.

3.2 Toxicological data

Most of the critical toxicological studies of ethion (Table 4) do not comply with GLP as the data were generated before the implementation of GLP standards. Overall, however, the Committee considered that the database was adequate to assess the risks of ethion.

Industrial Bio-Test Laboratories (IBT Labs) generated a substantial amount of data from toxicological studies of ethion. Because IBT Labs were subsequently found to have engaged in scientific misconduct, a joint programme called the IBT Review Program, led by the USEPA and Health and Welfare Canada (now Health Canada), re-examined the validity of the toxicological effects of many pesticides, including ethion, in 1983. Only those IBT studies of ethion that were validated by the IBT Review Program were included in the Committee's deliberations.

Table 4
Studies relevant to risk assessment

Species/study (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				
Two-year and carcinogenicity study (diet)	Males: 0, 0.11, 0.22, 1.17 Females: 0, 0.12, 0.24, 1.28	Erythrocyte AChE inhibition	1.17 a	_
Rat				
Thirteen-week studies	Study 1: 0, 0.15, 0.5, 1.5, 5.0 Study 2: 0, 15, 30, 50, 75	Erythrocyte AChE inhibition	0.15	0.5
Two-year and carcinogenicity study (diet)	Males: 0, 0.09, 0.18 1.8 Females: 0, 0.11, 0.22, 2.2	Erythrocyte AChE inhibition	1.8 [a]	_
Three-generation reproductive toxicity study (diet)	0, 0.1, 0.19, 1.2	Erythrocyte AChE inhibition	1.2 [a]	_
Developmental toxicity study (gavage)	0, 0.2, 0.6, 2.5	Delays in ossification of the ischium and pubes of the pelvis, and hyoid and manubrium	0.2 *	0.6
Dog				
Thirteen-week study (diet)	Males:0, 0.01, 0.06, 0.7, 6.9 Females:0, 0.01, 0.07, 0.71, 8.3	Brain AChE inhibition	0.06	0.71
Human				
Three-week study of effects of ethion on erythrocyte AChE activity (oral) [b]	0, 0.05, 0.075, 0.1, 0.15	Erythrocyte AChE inhibition	0.15**, [a]	_

* Pivotal study value for the derivation of the ADI (*Hoberman, Christian & Christian, 1983*).
** Pivotal study value for the derivation of the ARfD (*Palazzolo, Fancher & Calandra, 1970*)
[a] Highest dose tested.
[b] Group dosed with 0.15 mg/kg bw received ethion for 3 days only.

Overall, the Committee considered that the database was adequate to assess the risks of ethion.

Ethion had some acute toxicity when given to rats by subcutaneous, intraperitoneal and dermal routes; however, it was of high acute toxicity when given to mice and rats by the oral route. The oral LD_{50} for ethion in female rats was cited by JMPR (FAO/WHO, 1969) as 27 mg/kg bw, and ranged from 65 to 97 mg/kg bw in males (May & Baker, 1960; Gaines, 1969). No evaluation of the AChE activity was performed in any of the single-dose studies.

Ethion was not irritating to the skin of rabbits (Weiner, 1985). Ethion did not induce skin sensitization in a study in guinea-pigs (Freeman, 1984).

The most sensitive effect observed in all species given repeated doses of ethion was inhibition of cholinesterase activity.

In a 13-week study, male and female rats received diets containing ethion at concentrations of 0, 300, 600, 1000 or 1500 mg/kg feed (equal to 0, 15, 30, 50 and 75 mg/kg bw per day). Complete inhibition of erythrocyte cholinesterase activity

occurred in all test groups except in males at 15 mg/kg bw, where inhibition was marked but not complete. No treatment-attributable gross or histopathological changes were observed in any of the surviving rats (Keller & Paynter, 1958).

Based on the decrease in erythrocyte AChE activity in both rat studies, the NOAEL was 0.15 mg/kg bw per day.

In a 13-week study in dogs, ethion was given in the diet at doses of 0, 0.5, 2.5, 25 or 300 mg/kg feed (equal to 0, 0.01, 0.06, 0.71 and 6.9 mg/kg bw per day for males and 0, 0.012, 0.07, 0.71 and 8.25 mg/kg bw per day for females, respectively). Reductions in brain and erythrocyte AChE activity at weeks 5, 9 and 13 were seen at 25 mg/kg feed. There were no adverse ophthalmological, haematological or histopathological changes. The NOAEL was 0.06 mg/kg bw per day based on inhibition of the brain AChE activity at 0.71 mg/kg bw per day (Bailey, 1988).

In a chronic toxicity and carcinogenicity study, male and female mice received ethion 0, 0.75, 1.5 or 8 mg/kg feed (equal to 0, 0.11, 0.22 and 1.17 mg/kg bw per day for males and 0, 0.12, 0.24 and 1.28 mg/kg bw per day for females, respectively) for 105 weeks. No effects on body weight, feed consumption, incidence of mortality, haematology, blood biochemistry or erythrocyte or brain cholinesterase were attributable to the administration of ethion. Incidences of tumours were not affected by treatment. Based on the absence of changes in erythrocyte AChE activity in all dose groups, the NOAEL was 1.17 mg/kg bw per day, the highest dose tested (Morrow, 1985).

In a 24-month chronic toxicity and carcinogenicity study, male and female rats were given diets containing 0, 2, 4 or 40 mg/kg feed (equal to 0, 0.09, 0.18 and 1.8 mg/kg bw per day for males and 0, 0.11, 0.22 and 2.2 mg/kg bw per day for females, respectively). There was a treatment-related decrease of serum cholinesterase values in both male and female rats at the highest dose, indicating sufficient exposure and bioactivation of ethion; however, there were no significant differences between treated and control groups in erythrocyte and brain AChE activity. Haematological, blood biochemistry and ophthalmological examinations found no treatment-attributable effects. Incidences of tumours were not affected by treatment (Morrow & Mayhew, 1985). Based on the absence of changes in erythrocyte AChE activity in all dose groups, the NOAEL was 1.8 mg/kg bw per day, the highest dose tested.

The genotoxic potential of ethion was investigated in an adequate range of in vitro and in vivo assays.

The Committee concluded that ethion was unlikely to be genotoxic.

The absence of any toxic effect elicited by the doses used in both mouse and rat chronic toxicity studies (maximum doses tested were 1.17 and 1.8 mg/kg bw per day, respectively) suggested to the Committee that an insufficient dose range had been used to adequately assess the carcinogenicity of ethion. However,

the Committee also considered the margins of exposure for median and 95th percentile consumers to ethion of 33 400 and 4270, respectively. In view of the lack of genotoxicity and the large margin of exposure, the Committee concluded that ethion is unlikely to pose a carcinogenic risk to humans from residues in the diet.

In a three-generation reproduction study, rats received diets containing ethion at 0, 2, 4 or 25 mg/kg feed (equal to 0, 0.1, 0.19 and 1.2 mg/kg bw per day). No effect was observed on erythrocyte AChE in any group. Gross pathological examination of F_0, F_1 and F_2 parental animals and their progeny showed no alterations attributable to the administration of ethion at any dose level (Salamon, Enloe & Mayhew, 1985). Based on the absence of changes in erythrocyte AChE activity, the NOAEL was 1.2 mg/kg bw per day, the highest dose tested.

In a developmental toxicity study, rats were dosed orally by gavage with ethion at doses of 0, 0.2, 0.6 or 2.5 mg/kg bw per day on gestation days 6 through 15. Treatment-related increased incidences of delayed ossification of pubes were observed in fetuses in the 0.6 and 2.5 mg/kg bw per day groups. There were no significant differences between treated and control groups in maternal body-weight gain or feed consumption. The NOAEL for developmental and reproductive toxicity was 2.5 mg/kg bw per day, the highest dose tested. Based on skeletal variations, the NOAEL for embryo/fetal toxicity was 0.2 mg/kg bw (Hoberman, Christian & Christian, 1983).

The Committee concluded that ethion was unlikely to be teratogenic in rats.

In a study to determine the potential of ethion to cause delayed neurotoxicity, four groups of 10 chickens received a single gavage dose of 2792 mg/kg bw of ethion in corn oil after protection from acute cholinergic effects with 10 mg/kg bw atropine given intramuscularly, and were observed for 21 days. Preliminary experiments determined this dose to be equal to the LD_{50} for ethion in chickens with atropine prophylaxis. A positive control group received 500 mg/kg bw of TOCP, a known delayed neurotoxic agent, in corn oil, and the negative control group received the vehicle only. Acute cholinergic signs were observed in the treated groups; 14 of the 40 dosed chickens died. However, after recovery from the acute effects, no clinical or histopathological signs of delayed neurotoxicity were observed in the treated groups. Evidence of delayed neurotoxicity was observed in the TOCP-treated chickens (Roberts et al., 1986).

The Committee concluded that ethion does not induce delayed neurotoxicity.

3.3 Observations in humans

A study in humans reviewed by JMPR (FAO/WHO, 1972), USEPA and ATSDR evaluated the effects of ethion on blood cholinesterase activities. Six male volunteers were given ethion in corn oil solutions in three divided doses (at 9:00, 12:00 and 17:00) of 0.05, 0.075, 0.1 or 0.15 mg/kg bw per day via gelatine capsule for 3 weeks at each dose level, except for the highest dose (0.15 mg/kg bw), which was given for 3 days only. Plasma and erythrocyte cholinesterase activities were determined 5 times during the 2 weeks of the pretest period and 3 weeks of the test period. No adverse clinical signs (blood pressure, pulse rate, pupil size, light reflex, eye accommodation, chest sounds, muscle tone, knee-jerk, tongue tremor and finger tremor) were observed. Although decreases in plasma cholinesterase were observed at 0.1 and 0.15 mg/kg bw dose levels, no statistically significant depression in erythrocyte AChE was observed at any time during the study (Palazzolo, Fancher & Calandra, 1970). Based on the absence of absence of effects on erythrocyte AChE activity at any dose tested, the NOAEL was 0.15 mg/kg bw, the highest dose tested.

3.4 Microbiological data

Considering the chemical structure and mode of action of ethion, the Committee did not anticipate any adverse effects of ethion residues on human gastrointestinal microbiota.

4. Evaluation

In order to establish an acceptable daily intake (ADI), the Committee considered four studies: (1) the human study, from which a NOAEL of 0.15 mg/kg bw per day was derived based on erythrocyte AChE inhibition; (2) the dog study, from which a NOAEL of 0.06 mg/kg bw was derived based on inhibition of brain AChE; (3) the 13-week rat toxicity study, from which a NOAEL of 0.15 mg/kg bw per day was derived based on inhibition of brain and erythrocyte AChE; and (4) the rat developmental toxicity study, from which a NOAEL of 0.2 mg/kg bw per day was derived based on embryotoxic effects.

The Committee compared the NOAELs for erythrocyte cholinesterase inhibition by ethion in rats (0.15 mg/kg bw per day), dogs (0.06 mg/kg bw per day) and humans (0.15 mg/kg bw per day). The Committee considered the dog study inappropriate to evaluate the effects of ethion on cholinesterase inhibition because dogs show considerably greater sensitivity to ethion than humans. Although the

Committee considered the repeated-dose human study of AChE inhibition more appropriate than the corresponding 13-week rat study for evaluating this end-point, the Committee did not consider the data from the human study sufficient to assess the non-cholinergic effects of ethion. Hence, an ADI of 0–0.002 mg/kg bw was established based on the rat developmental toxicity study, using the NOAEL of 0.2 mg/kg bw per day and a safety factor of 100.

The Committee established an acute reference dose (ARfD) of 0.02 mg/kg bw, based on the NOAEL of 0.15 mg/kg bw for erythrocyte AChE inhibition in the repeated-dose human study and using a 10-fold intraspecies safety factor. The Committee acknowledged that the ARfD was likely conservative, since an acute oral lowest observed-adverse-effect level (LOAEL) for ethion has not been identified in humans.

As the ADI was based on developmental effect and is appreciably lower than the ARfD, there is a potential concern for exposure in pregnant women. Therefore, exposure in high-percentile pregnant consumers or a suitable surrogate population should be addressed. This exposure scenario will also be protective of children given the nature of the end-point on which the ADI is based.

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Flumethrin

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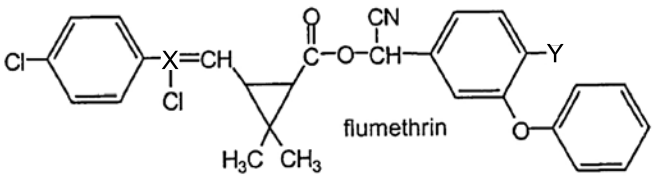
1. Explanation

Flumethrin, the common name for (*RS*)-cyano-4-fluoro-3-phenoxybenzyl 3-(β,4-dichlorostyryl)-2,2-dimethylcyclopropanecarboxylate) (International Union of Pure and Applied Chemistry), with the Chemical Abstracts Service number 69770-45-2 (Fig. 1) is an α-3-phenoxyphenyl pyrethroid (type II) insecticide. Flumethrin has four centres of optical or steric isomerism. Current flumethrin-containing products consist of more than 90% *trans*-Z-1 and *trans*-Z-2 isomers, with minor contributions from *cis*-Z (<2%) and *trans*-*E* (<1%) isomers.

Flumethrin is used in the control of ectoparasites on food-producing and companion animals as pour-on treatments, sprays or dips. It is also used as impregnated plastic strips for treatment of honey bees.

Fig. 1.
Structure of flumethrin



Positions of radiolabels: X = [Cl-phenyl-U-^{14}C]flumethrin; Y = [F-phenyl-U-^{14}C]flumethrin

Flumethrin has not been previously evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA), but has been previously evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) at its meeting in 1996, when an acceptable daily intake (ADI) of 0–0.004 mg/kg body weight (bw) was established, on the basis of the no-observed-adverse-effect level (NOAEL) of 0.36 mg/kg bw per day in a two-generation study of reproductive toxicity in rats (FAO/WHO, 1996).

The Committee evaluated flumethrin at the present meeting at the request of the Codex Committee on Residues of Veterinary Drugs in Food, with a view to establishing relevant health-based guidance values and to recommend maximum residue limits for honey (FAO/WHO, 2016).

Flumethrin is registered in several countries for the diagnosis and control in honey bee colonies of varroatosis (also known as varroosis), a disease caused by the parasitic mite *Varroa destructor*. Usually a plastic (low-density polyethylene; LDPE) strip is impregnated with 3.6 mg flumethrin (0.5 mg/cm^3). This product is inserted between the combs in the brood chamber of the beehive. When used as recommended, no withdrawal period is required, although this is based on various restrictions.

This product is authorized for use in several Member States. The restrictions of use are as follows:

- The product should be used after the honey is harvested, usually in late summer. It should not be used during the period of honey

flow. For use as a diagnostic tool, or in cases of severe infestation, the product can be used at any time of the year (all regions).

- In cases of treatment for high infestation during honey flow periods, the comb honey should not be sold (one Member State only; this is not stated directly in other regions, but it is implied).
- The usual dose recommended in all regions where the between comb product is authorized is four strips per chamber in developed colonies and two strips per chamber in young colonies. The strips are suspended in the spaces between the combs in the central broodrearing area. The strips should remain in the colony for 24 hours (diagnosis) or for 4–8 weeks (treatment), although the most common recommendation is for 6 weeks.

Another type of beehive strip, based on polyvinyl chloride (PVC) rather than LDPE, has also been authorized in some Member States. Instead of being inserted between the honey combs, this type of beehive strip is used as a gate at the entrance of the beehive.

The recommended dose for the beehive gate strips (275 mg flumethrin per strip) is two strips per beehive. The gate strips are fitted to the entrance of the hive so that the bees are forced to use the holes in the strips (15 per strip) to enter or leave the hive. The recommended duration of application is between 9 weeks and 4 months, just after honey flow and extraction.

Information submitted to the Committee included new studies on acute toxicity, short-term and long-term toxicity, reproductive and developmental toxicity, genotoxicity, carcinogenicity and neurotoxicity that had not been previously considered by JMPR in 1996. Pivotal studies were, in general, conducted according good laboratory practice (GLP) standards.

A literature search was performed using the search term "flumethrin". The following databases were searched on 5 May 2017: PubMed and ToxNet (United States National Library of Medicine, National Institutes of Health, USA). Of the 12 additional papers identified in this search, none provided any information on the toxicology of flumethrin additional to that assessed by JMPR in 1996) or additional to data submitted under the current assessment.

2. Biological data

2.1 Biochemical aspects

2.1.1 Absorption, distribution and excretion

(a) Oral administration

(i) Rats

In a single oral dose study reported by JMPR in 1996, about 30% of a dose of [F-phenyl-U-^{14}C]flumethrin given to rats was absorbed (Steinke, Weber & Suwelack, 1983).

Klein (1993a) investigated the kinetics and metabolism of flumethrin (purity 98.2% for the *trans-Z*-1 isomer and 97.3% for the *trans-Z*-2 isomer) in a GLP-compliant study. Rats (Wistar) were administered [Cl-phenyl-U^{14}C] flumethrin in physiological saline solution containing 5% Cremophor. The dosing groups were as follows: 1 mg/kg bw of a single oral dose to male and female rats; 5 mg/kg bw of a single oral dose to male rats; 1 mg/kg bw of an oral dose repeated for 7 days to male rats; and 1 mg/kg bw of a single intraduodenal dose to bile duct–fistulated male rats. Analyses were conducted up to 7 days after dosing.

Absorption was rapid but incomplete. Approximately 75% of the dose was absorbed, with 77–88% eliminated in the faeces mostly after absorption and excretion in the bile. Only 2% was excreted in urine (with approximately 68% eliminated in faeces in the first 24 hours after a single dose and only 2% via urine). After 7 doses, the proportion of faecal excretion dropped to 37% and of urinary excretion dropped to 1.7%, resulting in an increase in residual radioactivity in the body. In bile duct–fistulated rats, 43% of the radioactivity from an intraduodenal dose (1 mg/kg bw) was excreted into the bile and 10% into urine, with approximately 20% unabsorbed in faeces. The highest levels of radioactivity were found in the plasma and lowest in the brain.

The difference in absorption values in this study, 75%, compared with 30% reported by Steinke, Weber & Suwelack (1983), is a result of the position of the radiolabel and may be due to ester hydrolysis in the stomach.

The time for plasma concentrations to increase from 25% to 75% of the maximal values (t_a) varied between 2 and 3.5 hours under the different test conditions, with the maximum achieved in about 8 hours. The elimination half-lives were 130–160 hours, demonstrating the slow release of radiolabel from the plasma; this was also reflected in the low plasma clearance rates (≤12 mL/kg bw per hour) and very low renal clearance rates (≤1.2 mL/kg bw per hour). The concentrations of radiolabel in the organs 48 hours after dosing were 3- to 50-fold lower than in plasma. Low concentrations were found in spleen, fat, brain and bone. The distribution volume under steady-state

conditions was 25–44% of the body volume, indicating either slow or limited distribution to peripheral compartments from plasma (considered as the central compartment). Redistribution into plasma before biliary excretion was slow, as indicated by the relatively large mean residence times (190–235 hours). The radiolabel accumulated in plasma after repeat dosing, so that the relative plasma concentration had increased almost 10-fold after 7 days. Once dosing stopped, the plasma concentration of radiolabel fell very slowly, the elimination half-life being about 155 hours. Seven days after administration, 9–20% of the dose was still present in the body, except the gastrointestinal tract) (Klein, 1993a).

In a GLP-compliant study, Klein (1993b) investigated the distribution of [Cl-phenyl-U^{14}C]flumethrin (purity 97.3–98.2% for the two *trans* isomers) by whole-body autoradiography in rats (Wistar, male) at 1, 4, 8, 24 and 48 hours after a single oral dose of 5 mg/kg bw.

The distribution of radioactivity after 1 hour demonstrated a fast onset of absorption from the gastrointestinal lumen, with the distribution pattern consistent throughout the study period. Elimination of radioactivity from the blood was delayed. The highest intensity of radioactivity in tissues other than the stomach was in the liver. High intensities were also found in spleen, kidneys, lungs, adrenal cortex, cartilage, bone marrow, pineal gland, pituitary glands and subcutaneous adipose tissue. The lowest intensities were found in the central nervous system (CNS).

The kinetics of flumethrin were studied as part of repeated-dose toxicity studies, and are reported here.

In a GLP-compliant 13-week subchronic oral toxicity study, rats (Wistar; 10/sex per dose) were administered oral doses of flumethrin at 0, 50 or 160 mg/kg feed in the diet as either the standard liquid (Bayticol P; equal to 0, 3.7 and 12.2 mg/kg bw per day for males and 0, 4.3 and 12.8 mg/kg bw per day for females, respectively) or granular formulation (Bayticol P Granulate; equal to 0, 3.5 and 11.8 mg/kg bw per day for males and 0, 4.5 and 12.9 mg/kg bw per day for females, respectively). Retro-orbital blood samples were taken at 1, 2, 3, 6, 9, 12 and 13 weeks during the study for analysis of flumethrin levels only.

The results indicated that the bioavailability of the two formulations did not differ significantly from each other at 160 mg/kg feed (equal to 12.2 and 12.8 mg/kg bw per day for Bayticol P, and 11.8 and 12.9 mg/kg bw per day for Bayticol P Granulate, for male and females rats, respectively). No data for the 50 mg/kg feed dose were provided (Andrews, 2000).

In a 15-week subchronic oral toxicity study in rats (5/sex per dose), Bomann & Sander (1995) administered flumethrin (purity 94.6%) at 0, 10, 40 or 160 mg/kg feed in the diet (equal to 0, 0.7, 2.9 and 11.9 mg/kg bw per day for males and 0, 0.8, 3.4 and 13.0 mg/kg bw per day for females, respectively). Blood samples were taken at weeks 5 and 13 and assessed for serum flumethrin levels.

The level of quantitation (LOQ) was 0.05 mg/L. Mean recovery rates were 74–83%. No flumethrin was detected in serum at 0 mg/kg feed and levels were below the LOQ for the animals treated at 10 mg/kg feed. At 40 mg/kg feed, flumethrin levels ranged from <0.05 to 0.172 mg/L at week 5 and <0.05 to 0.08 mg/L at week 13. At 160 mg/kg feed, flumethrin levels ranged from 0.069 to 0.376 mg/L at week 5 and <0.05 to 0.557 mg/L at week 13. Values include both sexes and were not corrected for recovery rates (Krebber, 1994).

In an exploratory subchronic toxicity study (study report not provided), rats (3/sex per dose) were administered flumethrin (purity 96.7%) at 0, 1, 5 or 50 mg/kg feed in the diet (equivalent to 0, 0.1, 0.5 and 5 mg/kg bw per day, using conversion values described in *Environmental Health Criteria 240: Principles and methods for the risk assessment of chemicals in food*; IPCS, 2009) for 4 weeks. Blood samples were taken at days 25/26 (at 4- to 6-hourly intervals for 24 hours) and assessed for plasma flumethrin levels. The LOQ was 25 µg/L, and the recovery rate was 78 ± 9.1%. Flumethrin levels were below the LOQ in the 0, 1 and 5 mg/kg feed dose groups. At 50 mg/kg feed, the maximum flumethrin plasma concentration was 62 ± 10 µg/L in males at 0:00 hours and 91 ± 58 µg/L in females at 20:00 hours. Values were not corrected for recovery rates (Krebber, 2006a).

In an exploratory subchronic toxicity study for a subsequent two-generation reproductive toxicity study (see section 2.2.5(a)), rats (3/sex per dose) were administered flumethrin (purity 95%) by gavage at 0, 0.08, 0.4, 2.0, 3.0 4.0 or 5.0 mg/kg bw per day. Retro-orbital blood samples were taken on days 59/60, 73, 92/93 and 106 (at various time points after dosing). The blood samples were assessed for plasma flumethrin levels.

The LOQ was 25 µg/L, and the recovery rate was 90 ± 10.5%. Flumethrin levels were below the LOQ for control and low-dose animals at 0.08 mg/kg bw. Flumethrin levels varied in the other treatment groups, but in general, peak concentrations were observed 4 hours after dosing and levels tended to be higher in male than female rats (Krebber, 2006b).

Blood samples were collected on days 38/39 in the pre-mating period of a two-generation reproductive toxicity study (section 2.2.5(a); Eiben, 2008) in F_0 rats (3/sex per dose) administered flumethrin (purity: 95.8%) by gavage at 0, 0.5, 1.0 or 3.0 mg/kg bw per day. Plasma flumethrin levels were assessed at 1, 2, 4, 7 and 24 hours after dosing.

The LOQ was 25 µg/L, and the recovery rate was 86 ± 5.7%. Flumethrin levels from the control group were all less than the LOQ, and all 24-hour samples in the treated groups were also less than the LOQ. As shown in Table 1, plasma flumethrin C_{max} doubled with an increase in dose from 0.5 to 1.0 mg/kg bw per day, but increased by less than 2-fold with an increase in dose from 1.0 to 3.0 mg/kg bw per day. The time to reach peak plasma concentration (T_{max}) was 4 hours

Table 1
Toxicokinetic parameters in rats in two-generation reproductive toxicity study (first analysis and reanalysis) [a]

	Measure per gavage dose of flumethrin					
	0.5 mg/kg bw		1.0 mg/kg bw		3.0 mg/kg bw	
Parameter	Male	Female	Male	Female	Male	Female
First analysis [a]						
C_{max} (µg/L)	171	105	339	260	508	492
T_{max} (h)	4	4	4	4	4	4
Reanalysis [b]						
C_{max} (µg/L)	168	93.0	310	227	499	489
$C_{max,\ normalized}$ (kg/L)	0.335	0.186	0.310	0.227	0.166	0.163
T_{max} (h)	4	4	4	4	4	4
AUC_{0-tn} (µg·h/L)	668	181	1 189	772	1 954	1 505
$AUC_{0-tn\ normalized}$ (kg·h/L)	1.34	0.362	1.19	0.772	0.651	0.502
$AUC_{0-24\ h}$ (µg·h/L)	811	nc	1 308	814	2 129	1 589
$AUC_{0-24\ h,\ normalized}$ (kg·h/L)	1.62	nc	1.31	0.814	0.710	0.530

AUC: area under the plasma concentration–time curve; AUC_{0-ttn}: area under concentration-time curve up to last nonzero value; bw: body weight; C_{max}: peak plasma concentration; LOQ: level of quantitation; nc: not calculated; T_{max}: time to reach C_{max}
[a] Control group flumethrin levels all less than the LOQ of 25 µg/L.
[b] 50% of the LOQ for flumethrin levels below the LOQ in the original analysis substituted so that at least two thirds of the levels were above the LOQ of 25 µg/L.
[c] Calculated by extrapolation.
Sources: Hafner & Mueller (2007); Krebber & Hoffend (2007)

for all treatment groups. Plasma flumethrin levels were generally higher in male than in female rats (Krebber & Hoffend, 2007).

This analysis was repeated, substituting 50% of the LOQ for flumethrin levels below the LOQ of the original analysis, when at least two thirds of the levels were above the LOQ. This allowed further pharmacokinetic parameters to be calculated. As shown in Table 1, there were slight sex-related differences in plasma C_{max} and area under the plasma concentration–time curve (AUC) values, with values in males slightly higher than those in females. T_{max} (4 hours) was unaffected in this reanalysis (Hafner & Mueller, 2007).

(ii) Rabbits

Başçi & Eraslan (2014) administered flumethrin (purity 95.5%) to rabbits (New Zealand White; 6 months old) at 0 or 10 mg/kg bw as a single intravenous dose and subsequently as a single oral (gavage) dose. The vehicle was dimethylsulfoxide/deionized water, 7 : 2 volume per volume (v/v). Serial blood samples were taken up to 72 hours after dosing to determine serum flumethrin levels.

The LOQ was 0.025 µg/mL. Table 2 summarizes the key toxicokinetic parameters. Bioavailability was 60.9%.

Table 2
Summary of toxicokinetic parameters[a] of flumethrin after oral and intravenous administration in rabbits

	Route of administration	
Parameter	**Intravenous**	**Oral**
$t_{½\beta}$ (h)	34.0 ± 4.2	43.3 ± 8.6
MRT (h)	48.0 ± 5.8	59.7 ± 10.5
$AUC_{0-\infty}$ (mg·h/mL)	36.1 ± 5.3	22.0 ± 2.0
C_{max} (µg/mL)	–	0.54 ± 0.09
T_{max} (h)	–	5.42 ± 0.97
Bioavailability (%)	–	60.9

$AUC_{0-\infty}$: area under the plasma concentration–time curve from time 0 to infinity; bw: body weight; C_{max}: peak plasma concentration; LOQ: level of quantitation; MRT: mean residence time; T_{max}: time to reach C_{max}; $t_{½\beta}$: half-life during the β-phase
[a] LOQ = 0.025 µg/mL.
Source: Başçi & Eraslan (2014)

(b) Dermal application

(i) Dogs

Jons & Krebber (2000) treated dogs (beagle; 4 males, 8 females; 11.3 ± 1.9 kg bw) topically (entire body surface sprayed with the lay of the hair) with flumethrin as a 0.3% weight per volume (w/v) spray at 3 mL/kg bw (equal to 9.3 ± 0.2 mg/kg bw). Blood samples were taken prior to dosing and at various days up to 28 days after dosing. The blood samples were analysed for serum flumethrin levels. The LOQ was 2.5 µg/L. The recovery rate was 53 ± 11.2%.

Flumethrin serum levels were, in general, below the LOQ for most animals following administration of the spray. Four animals had flumethrin serum values of 0.028–0.051 mg/L at 12 hours post dose; at 24 hours post dose, the value was 0.034 mg/L in one animal and 0.123 mg/L in another animal. All values at day 0.25 and at day 4 and later were below the LOQ, and were corrected for recovery rates.

(ii) Sheep

Speirs & Donachie (1999) assessed the kinetics of [Cl-phenyl-^{14}C-flumethrin] in sheep (Suffolk Cross) after topical and intravenous administration in a GLP-compliant study.

Radiolabelled flumethrin at 3.3 mg/kg bw (target dose) was applied to the shaved skin of two male sheep and left in place for 1 hour under an occlusive dressing. The treated area was then washed with soap and water, and dried. One animal was killed at 24 hours and the other at 72 hours. Serial blood samples were taken over the 24/72-hour period after dosing; urine and faeces were also collected.

Measurable levels of radioactivity were observed in plasma approximately 6–8 hours after dosing, increasing with time until scheduled kill, with values up to 0.005 µg eq./mL in the first sheep (at 24 hours) and 0.009 µg eq./mL in the second sheep (at 48 hours). Approximately 1.7% and 3.0% of the administered dose from the first and second sheep, respectively, was recovered from the excreta and whole organs (liver and kidney). The highest concentration of total radioactivity was detected at 72 hours in fat (0.044 µg eq./g), although dosed skin contained 15.0 and 8.61 µg eq./g at 24 and 72 hours, respectively.

As most of the labelled dose was recovered from the skin wash and dressing, the dermal absorption through sheep skin was considered to be limited (Speirs & Donachie, 1999).

Further experiments after intravenous administration are described in section 2.1.1(c).

(iii) Cows

In a GLP-compliant study, Cameron & Phillips (1986) determined plasma and tissue distributions of [F-phenyl-^{14}C]flumethrin after topical application on a lactating cow (Fresian). Serial blood sampling was undertaken over 47 hours following dosing.

The LOQ was 1.9 ng eq./mL. Peak plasma radioactivity of 6.3 ng eq./mL was detected at 23 hours post dose. This fell to 4.1 ng eq./mL by 47 hours. Peak milk radioactivity of 3 ng eq./mL was detected at 31 hours post dose. At necropsy, radioactivity above the LOQ was detected in liver, kidney, gall bladder bile and urine (9, 10, 70 and 281 ng eq./mL, respectively). A significant amount (71.7% of the administered dose) was recovered at the application site and underlying skin.

(c) Intravenous administration

(i) Rabbits

Başçi & Eraslan (2014) administered flumethrin (purity 95.5%) at 0 or 10 mg/kg bw as a single intravenous dose and subsequently as a single oral (gavage) dose. The study is described in section 2.1.1(a) and key toxicokinetic parameters are reported in Table 2.

(ii) Sheep

Sheep (Suffolk Cross; 2/sex) received a single intravenous dose of 1 mg/kg bw of [Cl-phenyl-^{14}C]flumethrin and were killed 24 or 72 hours after dosing. Urine and faeces were collected during this period. The data from one male killed at 24 hours were not included in the overall assessment because a test substance–administration error resulted in inconsistent data.

Plasma radioactivity levels at 24 and 72 hours were 0.302 and 0.086 µg eq./mL, respectively. At 24 hours post dose, the liver accounted for 2.9% (1.321 µg eq./g) and the kidneys for 0.2% (0.392 µg eq./g) of the administered radioactivity. At 72 hours post dose, these tissues accounted for 0.4% (0.239 µg eq./g) and 0.02% (0.090 µg eq./g), respectively. At 24 and 72 hours post dose, fat (composite) contained 0.172 and 0.207 µg eq./g, respectively. Although no sex-related differences were noted, the small number of animals tested precludes any definitive conclusion (Speirs & Donachie, 1999).

Urine, liver and composite fat samples from the three intravenously dosed sheep in the Speirs & Donachie (1999) study underwent two different extraction methods prior to being assessed by high-performance liquid chromatography (HPLC). The major metabolite in urine was 3-[2-chloro-2-(4-chlorophenyl)ethenyl]-2,2-dimethylcyclopropanecarboxylic acid (flumethrin acid [BNF5533A]), present at 3.50–16.29%. Several other metabolites were detected but not identified. The major residue in liver was also flumethrin acid, at 26.1–58.8%. Unchanged flumethrin was also identified in liver samples (5.5–12.1%). Several minor unidentified metabolites in liver accounted for a maximum of 5% of the dose. Fat (composite) contained mostly flumethrin (37.1–66.0%), with additional unidentified components (1.2–13.7%) (Phillips, 1996).

2.1.2 Biotransformation

(a) Rats

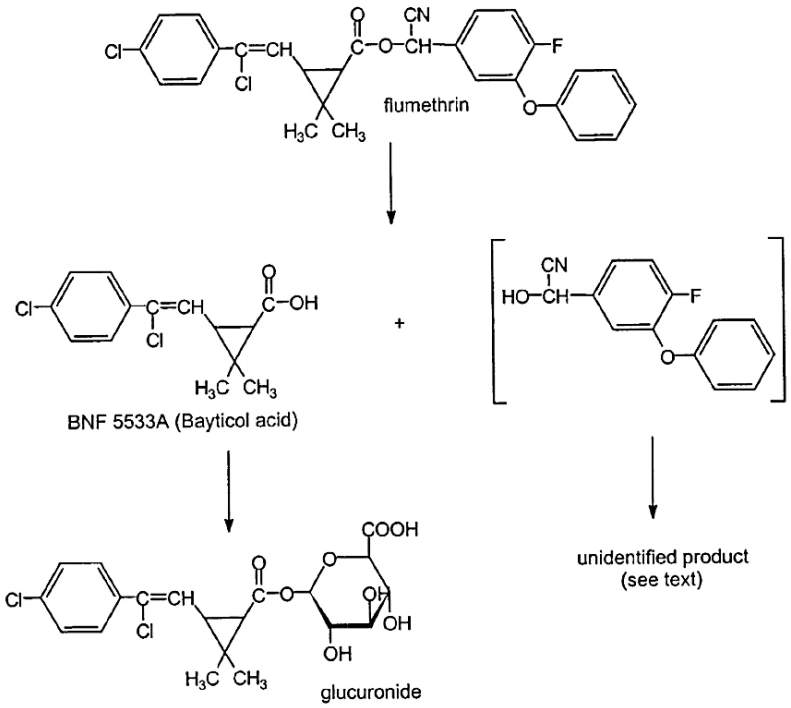
In the Klein (1993a) study (section 2.1.1(a)), the main radioactive compounds found in the faeces were unchanged flumethrin (which accounted for about 50% of the radiolabel recovered from male rats and about 25% of that from female rats) and the ester hydrolysis metabolite, flumethrin acid (which accounted for 15–18% of the radiolabel recovered from male rats and about 30% of that from female rats). No other biotransformation products were found in the faeces.

Following oral administration of [F-phenyl-U-^{14}C]flumethrin to male rats (Sprague Dawley) at 10 mg/kg bw, two primary metabolites were identified in urine. These were 3-(4-hydroxy-phenoxy)-4-fluorobenzoic acid and 3-phenoxy-4-fluorobenzoic acid, which accounted for 50% and 35%, respectively, of the urinary radiolabel over 0–24 hours and 80% and 10% of the urinary activity over 24–48 hours. Glycine conjugates (hippuric acids) of the primary metabolites were also identified, but each accounted for no more than 4% and 7.4%, respectively, of the urinary radiolabel (Ecker, 1983).

The proposed metabolic pathway of flumethrin is shown in Fig. 2.

Rats (Wistar; 12 males) were administered flumethrin at 40 mg/kg bw by intraperitoneal injection for 6 days to ascertain the effect of the compound on hepatic drug-metabolizing enzymes and antipyrine disposition. Results

Fig. 2.
Proposed metabolic pathway for flumethrin



Note: The "unidentified product" is further described in section 2.1.2(b).
Source: Klein (1993a, 1995)

indicate a type II pyrethroid response, where there were reductions in hepatic enzyme levels and catalytic activities of monooxygenases and the oxidation of antipyrine. In particular, there were reductions in microsomal total cytochrome P450 content (36%), NADPH–cytochrome c reductase activity (38%), aniline hydroxylase activity (53%), aminopyrine *N*-demethylase activity (54%) and uridine 5′-diphospho (UDP)-glucuronosyltransferase activity (34%). The total plasma clearance of antipyrine was reduced (54%), and the urinary excretion of norantipyrine, 4-hydroxyantipyrine and 3-hydroxymethyl antipyrine was reduced by 60%, 38% and 33%, respectively (Anadón et al., 1995).

(b) Cows

The metabolism of flumethrin was assessed in 1 dairy cow (Friesian) and 1 male beef cow (steer; Continental cross-breed) after a single intravenous 1 mg/kg bw dose of [Cl-phenyl-^{14}C]flumethrin. Radioactivity was measured in excreta and

edible tissues (kidneys, liver, muscle and fat – subcutaneous and omental) and milk 8 hours after dosing.

The recovery of radioactivity was 36% for the cow and 22% for the steer, with additional radioactivity likely retained in the animals' bodies. A small proportion of the labelled dose was excreted in the urine of the cow (4%) and the steer (8%) at 8 hours post dosing. Faecal and milk excretion were less than 1% of the administered dose at 8 hours post dosing. The highest levels of radioactivity were in liver: 21.1% of dose (12.98 µg eq./g) in the cow and 4.42% of dose (3.36 µg eq./g) in the steer. Levels in other tissues were much lower (<10% that of liver, other than in the kidney of the steer [~40%]) (Gifford & Dunsire, 1994).

Further analysis of these samples identified parent (flumethrin) and the metabolite flumethrin acid in all tissues. Milk contained flumethrin (68%) and an unknown metabolite (11.5%) but not flumethrin acid. The glucuronidated form of the acid (KNO 2107) was also detected in liver and kidneys (Klein, 1995).

2.2 Toxicological studies

2.2.1 Acute toxicity

(a) Lethal doses

The single-dose toxicity of flumethrin was investigated in rats using various vehicles for oral and dermal administration. The oral median lethal dose (LD_{50}) in rats was dependent on the vehicle used.

When flumethrin (range: 170–2677 mg/kg bw) was given in peanut oil, the LD_{50} was 911 and 662 mg/kg bw in male and female rats, respectively. At all doses, animals had unkempt fur, narrowing of the peripheral fissures, reduced motility, salivation, a strutting unsteady gait and a reduced gripping capacity. There were no deaths at 170 mg/kg bw (the lowest dose tested in both sexes) when observed for up to 5 days, but at 256 mg/kg bw, 3/10 males died 3–4 days after dosing and 3/10 females died 4–5 days after dosing (Renhof, 1983a).

On the other hand, when flumethrin (range: 400–8000 mg/kg bw) was given in miglyol (no further details provided; assumed to be a propylene glycol diester), the LD_{50} was 3849 and 2248 mg/kg bw in male and female rats, respectively. Similar clinical signs were observed as in the animals treated with flumethrin in the peanut oil vehicle. There were no deaths in males at 630 (the lowest dose tested in males) and 2000 mg/kg bw up to 6 days after dosing, but 2/10 at 1000 mg/kg bw died up to 4 days after dosing. In females, there were no deaths at 400 mg/kg bw (the lowest dose tested in females), but 1/10 at 630 mg/kg bw died 3 days after dosing. Irrespective of the vehicle, necropsies of animals that died revealed air- and fluid-filled stomachs and fluid-filled intestines (Renhof, 1983a).

When Cremophor EL (2%) emulsion, a polyethoxylated castor oil, was used as the vehicle, the oral LD_{50} was 56 and 41 mg/kg bw in male and female rats, respectively (Bomann, 1992).

In another study, the LD_{50} was greater than 100 mg/kg bw, the highest dose tested, in both male and female rats (Bomann, 1994a). This vehicle was considered an important contributor to the oral toxicity of these flumethrin formulations (Bomann, 1994b).

When acetone : peanut oil (1 : 10) was used as the vehicle, the oral LD_{50} for flumethrin (range: 63–1000 mg/kg bw) was 302 and 138 mg/kg bw in male and female rats, respectively. At all doses, animals had unkempt fur, narrowing of the palpebral fissures, reduced motility, salivation, a strutting unsteady gait and a reduced gripping capacity. Isolated cases also included prone position, increased lachrymation and diarrhoea. No males at 100 mg/kg bw (the lowest dose tested in males) died for up to 4 days, but 2/10 males at 160 mg/kg bw died up to 3–5 days after dosing. There were no deaths in females at 63 mg/kg bw (the lowest dose tested in females), but 2/10 females at 80 mg/kg bw died up to 3 days after dosing. Autopsy results for all animals that died also revealed air- and fluid-filled stomachs and fluid-filled intestines (Renhof, 1983b).

The oral LD_{50} for flumethrin in corn oil (50% v/v) in female rats was 175 mg/kg bw when tested using the up-and-down procedure at 55, 175 and 550 mg/kg bw. There were no deaths at 55 mg/kg bw. Clinical signs in the surviving female rats included decreased activity, crusted fur, diarrhoea, hunched posture, piloerection, polyuria and salivation, which were not evident by day 6 (Kuhn, 2007a).

The *trans-Z*-2 isomer was the key contributor to the acute oral toxicity in rats, with an LD_{50} value of between 10 and 50 mg/kg bw using Cremophor EL (2%) emulsion. In contrast, the oral LD_{50} values for the *trans-Z*-1 isomer was greater than 5000 mg/kg bw in male rats and greater than 500 mg/kg bw in female rats, the highest doses tested (Andrews, 1997).

The dermal LD_{50} for flumethrin (range: 500–4000 mg/kg bw) in corn oil (1 mL/g test substance) was 1998 and 1436 mg/kg bw for male and female rats, respectively. No deaths occurred at 500 mg/kg bw (the lowest dose tested in both sexes). Clinical signs in survivors included decreased activity, emaciation, hunched posture, lateral recumbency, nasal discharge, piloerection, polyuria, salivation, spots around the eyes, stained fur and sensitivity to touch/sounds through to day 14. Body tremors, crusted eyes, diarrhoea, swollen feet and self-mutilation were observed only in those animals that died (Kuhn, 2008). Similar results were observed in another study using corn oil as the vehicle, with the LD_{50} greater than 2000 mg/kg bw in both male and female rats (Bomann, 1994c).

The median lethal concentration (LC_{50}) for flumethrin was 0.572 mg/L in air for male and female rats. Details are given in section 2.2.2(c), on inhalational toxicity (Pauluhn, 1996).

Additional information on the single-dose toxicity of flumethrin in various formulations is available in the JMPR review (FAO/WHO, 1996).

(b) Dermal irritation

The skin-irritating potential of flumethrin (purity not stated) was investigated in three rabbits (New Zealand White) in a GLP-compliant study. The drug was applied to intact clipped skin on the dorsal–lateral area of each rabbit, while a contralateral area was used for testing the control (olive oil). The test substance (500 µL of 10% flumethrin formulation in olive oil) was applied to the skin under a patch of gauze held in place for 4 hours with a semi-occlusive dressing. Treated areas were washed with water and olive oil, and scored at 24, 48 and 72 hours and 7 days after removal of the patch.

Under the conditions of the test, there was no evidence of skin irritation by flumethrin (Krötlinger, 1994).

In another GLP-compliant study, Kuhn (2007b) investigated the skin-irritating potential of flumethrin (purity 95.8%) in three rabbits (New Zealand White). Flumethrin was applied to intact clipped skin on the dorsal area of each rabbit, while a contralateral area was used for testing the control (corn oil). One mL of the test mixture (0.5 mL flumethrin and 0.5 mL corn oil) was applied to the skin under a patch of gauze held in place for 4 hours with a semi-occlusive dressing. Treated areas were washed with tap water, and scored at 1, 24, 48 and 72 hours after removal of the patch.

Apart from very slight erythema at 1 hour in all three animals, flumethrin did not cause irritation to the skin under the conditions of the test (Kuhn, 2007b).

(c) Ocular irritation

The eye-irritating potential of flumethrin (purity not stated) was investigated in three rabbits (New Zealand White) in a GLP-compliant study. Krötlinger (1994) applied 100 µL of 10% flumethrin formulation in olive oil to one eye of each rabbit, while treating the other eye with control (olive oil). Eyes were rinsed with saline and scored at 1, 24, 48 and 72 hours and 7 days post-treatment.

There was slight reddening and/or swelling of the conjunctiva up to 48 hours in all three rabbits (in one case due to a wound under one eyelid), but this was reversible, indicating that under the conditions of the test flumethrin was not irritating to the eye (Krötlinger, 1994).

(d) Dermal sensitization

In a GLP-compliant study, flumethrin (purity 88.3%) was tested for dermal sensitization potential using the Magnusson and Kligman maximization test in male guinea-pigs (Bor:DHPW, i.e. Hsd/Win:DH). For intradermal induction, flumethrin was administered as a 5% solution in polyethylene glycol 400 (PEG400); for topical induction and challenge, it was administered as a 50% solution in PEG400.

There were no post-challenge skin reactions (Diesing, 1991).

When Vohr (1994) repeated this study with a different batch of flumethrin (purity 94.6%) but the same strain of guinea-pigs and the same PEG400 formulation and with an additional 25% concentration for challenge, only one animal in each treatment group (25% and 50%) showed slight partial skin reddening at 48 hours after the start of exposure, and not at 72 hours.

Under the conditions of the test, flumethrin was not considered to cause skin sensitization (Vohr, 1994).

2.2.2 Short-term studies of toxicity

(a) Oral administration

(i) Mice

In two GLP-compliant dose range–finding studies, mice (CD-1; 10/sex per dose) received flumethrin in the diet at either 0, 60, 120, 240 or 480 mg/kg feed or 0, 5 or 10 mg/kg feed for 3 months (equal to 0, 0.9, 1.9, 9.9, 20.8, 42.2 and approximately 80 mg/kg bw per day for males and 0, 1.4, 2.5, 13.2, 26.5, 56.0 and approximately 100 mg/kg bw per day for females). Purity of the flumethrin was 95.15% and 94.4%, respectively.

Mortalities in both sexes increased at 60 mg/kg feed and above, with all animals at 480 mg/kg feed dying within the first week of treatment. Cumulative survival for animals receiving 60, 120 and 240 mg/kg feed was 80%, 60–70% and 50–30%, respectively.

In the second study, two females in the control and 5 mg/kg feed groups died in week 13 and 10, respectively. Two other deaths, in week 13, were associated with blood sampling. The severity of clinical signs was dose dependent. At 5 mg/kg feed, there were a limited number of undefined skin changes in animals; at 10 mg/kg feed, there was also hair loss and piloerection. At 60 mg/kg feed, emaciation was also observed; and poor condition was noted at 120 mg/kg feed. At 240 mg/kg feed, both sexes showed signs of increased salivation, apathy, decreased reactivity, convulsions, tremor and high stepping gait/squatting position. Skin changes noted at 60 mg/kg feed evolved further into instances of injury, wounds and inflammation; these skin changes were considered to be a consequence of flumethrin-induced increased grooming and scratching behaviour because of

its effects on sensory neurons. These sequelae did not show themselves at 480 mg/kg feed because none of the animals lived long enough. Body-weight loss was evident in the first week at 120 and 240 mg/kg feed; this was followed by an increase in body weight although this was retarded at 240 mg/kg feed. Feed intake was reduced at the higher doses, particularly in females at 60 and 120 mg/kg feed (14% compared with controls) and 240 mg/kg feed (24% compared with controls). There was a tendency for lower water intake with dose, especially at 240 mg/kg feed, for both sexes.

Haematological effects were more pronounced at 120 mg/kg feed and above at week 13 in both sexes: lower leukocyte counts (males: 6.0 versus 7.2 $\times$ 10^9/L in controls; females: 4.3 versus 7.3 $\times$ 10^9/L in controls); higher platelet counts at 120 mg/kg feed and above (e.g. 240 mg/kg feed: males: 1497 versus 1319 $\times$ 10^9/L in controls; females: 1470 versus 1076 $\times$ 10^9/L in controls); and reduced packed cell volume at 240 mg/kg feed (males: 46.0 versus 47.4; females 46.4 versus 47.6 in controls). A reduction in lymphocytes (e.g. 240 mg/kg feed males: 53.6% versus 81.6% in controls; 240 mg/kg feed females: 67.2% versus 84.1% in controls) was accompanied by an increase in segmented neutrophils from 120 mg/kg feed (e.g. 240 mg/kg feed males: 44.9% versus 16% in controls; 240 mg/kg feed females: 32.2 versus 14.9% in controls). Similarly, changes in clinical chemistry parameters were noted at the higher doses in week 14; these included increases in blood protein (e.g. 240 mg/kg feed males: 62.3 versus 56.8 g/L in controls; females: 59.4 versus 57.0 g/L in controls); reduced glucose (e.g. 240 mg/kg feed males: 5.45 versus 6.66 mmol/L in controls; females: 5.04 versus 5.49 mmol/L in controls); and bilirubin (e.g. 240 mg/kg feed males: 1.3 versus 2.2 µmol/L in controls; females: 1.2 versus 2.0 µmol/L in controls). These changes in clinical chemistry parameters were most pronounced at 240 mg/kg feed.

Slight changes in some organ weights compared with controls were evident at the end of the study. These were more marked at 240 mg/kg feed than at other doses. For example, relative adrenal weight (males: 28 versus 16 mg/100 g bw; females: 38 versus 35 mg/100 g bw); relative spleen weight (males: 447 versus 424 mg/100 g bw; females: 819 versus 533 mg/100 g bw); relative testes weight (991 versus 737 mg/100 g bw); and relative ovary weight (79 versus 139 mg/100 g bw). However, the lack of treatment-related histopathological findings in these organs indicated that these weight changes were likely a result of the effects on body weight (240 mg/kg feed males: 27 versus 36 g in controls; females: 25 versus 30 g in controls). The key histopathological findings were related to skin ulcers that occurred in a dose-dependent manner in all treated groups. As noted by the pathologist, hyperkeratosis and acanthosis were observed adjacent to the ulcers, and in some cases, were related to findings in other organs, especially the thymus (cortical atrophy), spleen (lymphocyte depletion) and mandibular lymph nodes

(lymphocyte depletion). At 5 mg/kg feed, the only effect observed was undefined skin changes.

The overall NOAEL in these two dose range–finding studies in mice was 5 mg/kg feed (equal to 0.9 and 1.4 mg/kg bw per day for males and females, respectively) based on clinical signs observed at 10 mg/kg feed (equal to 1.9 and 2.5 mg/kg bw per day for males and females, respectively) (Leser & Popp, 1998).

(ii) Rats

In a non-GLP-compliant study, rats (Wistar Crl:(WI); 5/sex per dose) were administered flumethrin (purity 95%) by gavage, using corn oil as a vehicle, at 0, 5.0, 7.5 or 10 mg/kg bw per day for 30 days.

There were no unscheduled deaths during the treatment period. Clinical signs were limited to increased salivation in mid- and high-dose males and females. Body weight was marginally depressed (5%) in high-dose females; this was more pronounced in mid-dose males from day 9 onwards and high-dose males from day 5 onwards. By study end, the body weights in these groups of males were 15% and 23% less than their respective controls. Nonsignificant reductions in feed intake were observed, in females in particular. Water intake was increased in mid- and high-dose males and females.

Significant decreases in absolute spleen weight (401 versus 611 g in controls), relative spleen weight (171 versus 2016 mg/100 g bw in controls) and absolute thymus weight (392 versus 675 g in controls) were noted in high-dose males. These were also seen at the other treatment doses (data not shown here). In addition, relative adrenal weight (22 versus 16 mg/100 g bw in controls) and relative testes weight (1212 versus 1045 mg/100 g bw in controls) were increased in the high-dose males. No significant organ weight changes were noted in females. The study did not include any histopathological assessments.

A NOAEL could not be identified in males. A NOAEL of 7.5 mg/kg bw per day was identified for females based on clinical signs and depressed body weight at 10 mg/kg bw per day (Eiben, 2006b).

Rats (Wistar BOR:WISW; 15/sex per dose) received flumethrin (purity not stated) in the diet at 0, 10, 50 or 150/250 mg/kg feed (150 mg/kg feed from the third week onwards) for 13 weeks (equal to 0, 0.70, 3.54 and 11.11 mg/kg bw per day for males and 0, 0.84, 4.21 and 13.24 mg/kg bw per day for females, respectively, with the highest concentration given being for 150 mg/kg feed). An additional set of rats (10/sex per dose) received the same doses and were killed at 4 weeks.

By 2 weeks, 10% of animals at 50 mg/kg feed and 50% of animals at 250 mg/kg feed treated showed inflammatory ulcerative skin changes. Unscheduled deaths occurred only at 250/150 mg/kg feed, with four females and one male

dying during the first 2 weeks of treatment and another male during week 5. Feed and water intake at 250/150 mg/kg feed were depressed, with accompanying body-weight loss in animals from both sexes. With a change in dose from 250 to 150 mg/kg feed from week 3 onwards, feed and water intake were comparable to the control group, and no further weight loss was evident. By scheduled kill at 13 weeks, body weight in this high-dose group was lower than in the control group by about 9% in males and 8% in females. No dose-dependent toxicological changes were noted in haematological measures, plasma and microsomal liver enzymes, serum electrolytes or urine analysis. Significant increases in relative brain weight observed in both males and females receiving 250/150 mg/kg feed were not associated with histopathological changes but were linked to changes in body weight compared with controls. Pathological findings in the brains of 3/4 high-dose animals that died prematurely included filled-in gyri and sulci regions of the brain, but there were no histopathological correlates.

The NOAEL was 10 mg/kg feed (equal to 0.7 mg/kg bw per day for males and 0.8 mg/kg bw per day for females) based on inflammatory ulcerative changes in the skin of animals treated at 50 mg/kg feed (equal to 3.54 mg/kg bw per day for males and 4.21 mg/kg bw per day for females) (Hahnemann & Rühl, 1985).

In a 15-week GLP-compliant study, rats (Wistar, BOR:WISW; 20/sex per dose) were given flumethrin (purity 94.6%) at 0, 10, 40 or 160 mg/kg feed in the diet (equal to 0, 0.7, 2.9 and 11.9 mg/kg bw per day for males and 0, 0.8, 3.4 and 13.0 mg/kg bw per day for females, respectively).

There were no unscheduled deaths during the study. Animals receiving 160 mg/kg feed exhibited significant skin lesions, reduced body weights and feed intake, piloerection, changes in motor activity and spastic or staggering gait. These animals were observed to groom themselves intensively and, in particular, to make scratching movements. For most of the animals in the high-dose group, this activity resulted in skin lesions, some of which were several centimetres in diameter and associated with bleeding. Two animals at 40 mg/kg feed also had skin lesions, but these were less intense than those on the high-dose animals and were generally reversible over the course of the study.

Body weights of animals at 160 mg/kg feed were 24% and 8% lower for males and females, respectively, by the end of the study, which was concordant with reduced feed intake in this group (males: 19.2 versus 28.0 g/animal per day in controls; females 14.9 versus 16.9 g/animal per day in controls). Similarly, increases in urinary density and reduced urinary volume were consistent with reduced water intake, but increased urinary protein may have been related to contamination from blood loss from skin lesions. Haematological changes at 160 mg/kg feed (haematocrit: −12%; haemoglobin: −14%; erythrocyte count: −16%; lymphocytes: −11%; segmented neutrophils: +145%) were consistent with blood loss from skin lesions and the poor condition of the animals. Changes in

clinical chemistry (bilirubin, protein, albumin) at 160 mg/kg feed were more likely the result of the poor condition of these animals, rather than a direct effect of flumethrin. The reduction in plasma cholesterol in high-dose males (24%) was not considered a direct effect of the treatment. There were no notable ophthalmological findings.

Gross pathological changes at necropsy were limited to skin lesions. Increased relative organ weights and some absolute organ weights noted in the high-dose group could be related to the significant reduction in body weight.

Histopathological findings in the high-dose group included evidence of extramedullary haematopoiesis stimulation in the spleen and a reduction in haemosiderin, considered to be related to blood loss via the skin lesions noted above. On the other hand, the reductions in the neutral fat content of liver and the size of the seminal vesicles in the high-dose animals were considered to be due to the poor condition of the animals and not due to flumethrin per se.

The NOAEL was 10 mg/kg feed (equal to 0.7 mg/kg bw per day for males and 0.8 mg/kg bw per day for females) based on isolated and reversible cases of scratching behaviour and skin lesions in animals treated with 40 mg/kg feed (equal to 2.9 mg/kg bw per day for males and 3.4 mg/kg bw per day for females) (Bomann & Sander, 1995).

(iii) Dogs

Dogs (beagle; 4/sex per dose; 8 months old) received diets containing flumethrin (purity not stated) at approximately 0, 50, 100 or 200 mg/kg feed (equal to 0, 2.1, 4.7 and 9.6 mg/kg bw per day for males and 0, 2.3, 5.0 and 9.0 mg/kg bw per day for females, respectively) for 13 weeks.

There were no unscheduled deaths. One high-dose female was euthanized in week 13 because water was inadvertently introduced into its lungs prior to collection of urine. Animals in all dose groups had thinning hair or hairlessness and, in some instances, weeping ulcerative scabbed patches on the neck, back, tail, ears and limbs. These lesions had partially healed by the end of the study. The high-dose group had reduced feed intake and body-weight gain and increased incidence of vomiting. Increased absolute and relative prostate weight in high-dose males were considered related to reduced body weight. Animals at the mid and high dose had slightly raised blood urea values compared with controls. There were no gross pathological changes in the kidney or liver.

A NOAEL could not be identified as the skin changes were noted in all dose groups (Hoffmann & Kaliner, 1984).

In a supplementary 13-week study, dogs (beagle; 4/sex per dose; 25 weeks old) received flumethrin (purity not stated) at 0 or 25 mg/kg feed (equal to 0 and 0.88 mg/kg bw per day for males and 0 and 0.94 mg/kg bw per day for females).

No differences were observed in adverse clinical signs, effects on feed/water intake, body weight, body temperature or haematological, clinical chemistry, urine analysis or ophthalmological parameters during the study, or organ weights or gross pathological changes at time of study termination. No histopathological examinations were performed as no histopathological differences were observed in the earlier Hoffmann & Kaliner (1984) study.

The NOAEL was 25 mg/kg feed (equal to 0.88 mg/kg bw per day for males and 0.94 mg/kg bw per day for females), in the absence of effects observed at this, the only dose tested (Hoffmann, 1985).

The overall NOAEL for flumethrin administered to dogs for 13 weeks was 25 mg/kg feed (equal to 0.88 mg/kg bw per day for males and 0.94 mg/kg bw per day for females) based on skin changes observed at 50 mg/kg feed (equal to 2.1 mg/kg bw per day for males and 2.3 mg/kg bw per day for females).

(b) Dermal application

In a non-GLP-compliant 2-week dermal toxicity study, female rats (Wistar; 3/dose) received flumethrin (95%) via a dermal patch to shaved skin at 0, 10, 30 or 100 mg/kg bw per day for 18 days. Two animals in each dose group had the test substance kept in place for 6 hours, whereas the third animal in each group had the test substance in contact for 24 hours. After 6 hours, the treatment area was washed with soap and water. In the first week, animals were treated for the first 5 days, but not on days 6 and 7. Subsequently, animals were treated every day. Another group (3 females) received 100 mg/kg bw per day for 6 consecutive days before termination.

There were no unscheduled deaths in any group. No adverse clinical observations were noted at 10 and 30 mg/kg bw per day. However, animals at 100 mg/kg bw per day had bloody nose, reduced motility, hunched back, uncoordinated gait, high stepping gait, laboured breathing, increased salivation, bleeding on noses and narrowed eyelids on several occasions. No significant skin reddening or skin thickness changes were noted in any group. The body weights of animals at 10 mg/kg bw per day were comparable to controls over the study period. At 30 mg/kg bw per day, there was a slight body-weight loss in the first week of treatment, but this was recovered subsequently. At 100 mg/kg bw per day, body-weight loss was noted in the first 4 days of treatment, but some body weight was subsequently recouped, although body weight was still less than that of the control animals at scheduled kill (184 versus 193 g, day 18). No statistical analysis was performed on body weights. No treatment-related changes were noted at necropsy.

The NOAEL was 10 mg/kg bw per day in female rats based on body-weight loss at 30 mg/kg bw per day within the first week of treatment (Schladt, 2007a).

In a non-GLP-compliant 4-week dermal toxicity study, rats (Wistar; 3/sex per dose) received flumethrin (purity 95.8%) via a dermal patch on shaved skin at 0, 100, 150 or 200 mg/kg bw per day, using corn oil as a vehicle, for 28 days. The test substance was kept in place for 6 hours, and the treated area was then washed with soap and water. The control group received corn oil only. The treated patch was administered every day for 5 days/week for the first 3 weeks. In week 4, the patch was applied for 7 days.

There were no unscheduled deaths in any group during the study. Flumethrin-treated animals had signs of bloody muzzle, sunken flanks, reduced motility, uncoordinated gait, high stepping gait, laboured breathing, diarrhoea, soft faeces, increased salivation and/or narrowed eyelids. Most of these signs were observed in the first week or first half of the study. Two females at 200 mg/kg bw had wounds smaller than 1 cm^2 on their flank or back. Skin reddening (low severity) was noted in both sexes at 150 mg/kg bw per day and in two females at 200 mg/kg bw per day. Skin thickness in treated males was slight reduced compared with controls, but no such changes were seen in the females. Body weight at 100 mg/kg bw per day developed similarly to controls. During the first few days, all other treated animals lost body weight but subsequently gained weight. Nonetheless, body weight at scheduled kill was less in males (8%, 10% and 17%) and females (20%, 25% and 25%) at 100, 150 and 200 mg/kg bw, respectively, compared with controls. Apart from the wounds identified on the two females at 200 mg/kg bw, there were no other necropsy findings at scheduled kill.

The NOAEL was 100 mg/kg bw per day based on skin reddening at 150 mg/kg bw per day (Schladt, 2007b).

In a GLP-compliant 13-week dermal toxicity study, rats (Wistar; 10/sex per dose) received flumethrin (purity 96%) via a dermal patch on shaved skin at 0, 1, 10, 30 or 80 mg/kg bw per day, using corn oil as vehicle for 93 days. The test substance was kept in place for 6 hours under a semi-occlusive dressing, and subsequently washed with soap and water. The control group received corn oil only. The treated patch was applied every day for 5 days/week for most of the study.

There were no unscheduled deaths in any group during the study. Both males and females at 30 mg/kg bw per day showed signs of uncoordinated gait and, for some, stilted gait. At the highest dose, reduced motility and high stepping gait were seen in both males and females. No toxicologically significant changes were noted in tests of motor activity or a functional observational battery (FOB) of tests. While some minor skin reddening was observed in high-dose males and

females during week 4, such changes were observed only occasionally and were also observed sporadically in other groups; they were therefore not considered toxicologically significant.

No significant changes in skin thickness or ophthalmological examinations were found. Body weight at 10 mg/kg per day was similar to that of controls. At 30 mg/kg per day, body-weight development was somewhat retarded and, at scheduled kill, body weights of males were 10% lower and of females were 6% lower than their respective controls. Body-weight loss was evident for the first few days in animals at 80 mg/kg bw per day. Subsequently, body weight increased during the study, but was nonetheless retarded; at study termination, body weights were 18% lower in males and 10% lower in females than their respective controls. While there were no significant dose-dependent changes in feed intake, water intake increased (body weight corrected) at 30 mg/kg per day (16% and 11% in males and females, respectively) and 80 mg/kg per day (26% and 20% in males and females, respectively) compared with controls.

Haematological changes were minor at 10 mg/kg per day, but at higher doses, reduced haemoglobin, haematocrit and erythrocyte levels were more evident, particularly in high-dose males (haemoglobin: 149 versus 157 g/L in controls; haematocrit: 0.466 versus 0.512 L/L in controls; 8.74 versus 9.57×10^{12}/L in controls). Similarly, reductions in creatine levels in both sexes (males: 52 versus 64 µmol/L in controls; females 57 versus 64 µmol/L in controls) and glucose levels in males but not females (males: 3.26 versus 3.65 mmol/L in controls; females 3.81 versus 3.49 mmol/L in controls) were more evident at the higher doses. On the other hand, cholesterol (1.82 versus 1.34 mmol/L in controls) and triglyceride levels (0.43 versus 0.29 mmol/L in controls) were elevated in high-dose females only. There were no toxicologically significant necropsy findings at termination and, apart from reductions in relative brain weight in high-dose males and females, no other significant findings were made. No skin lesions were noted in any of the treatment groups. Thymic atrophy was recorded in two males (overall incidence: 0, 0, 0 and 2) and five females (overall incidence: 2, 1, 3 and 5) at the high dose. Ovarian cysts (overall incidence: 2, 1, 3 and 5) were also seen in high-dose females.

The NOAEL was 10 mg/kg bw per day based on effects on body weight, water intake and haematology (erythrocyte numbers and haematocrit) at 30 mg/kg per day (Schladt, 2008).

(c) Exposure by inhalation

In a GLP-compliant study, rats (Wistar; 5/sex per dose) were exposed on a single occasion over 4 hours, via a nose cone, to a liquid aerosol of flumethrin (purity 94.4%) at 0, 0.048, 0.277. 0.309, 0.585 and 0.812 mg/L, using a 50 : 50 formulation

with PEG400. Mass median aerodynamic diameter (MMAD) was approximately 1.2 μm, and geometric standard deviation (GSD) was approximately 1.8 μm. Animals were observed for up to 2 weeks post exposure.

There were deaths in males at 0.309 mg/L and above and in females at 0.048 mg/L and above. Animals in all groups demonstrated a concentration-dependent increase in adverse clinical signs (e.g. piloerection, red encrustations around the nose and/or muzzle, bradypnoea, tremor, uncoordinated gait, salivation and prostration) and effects on body temperature (hypothermia). Animals in the higher exposure groups had decreased grip strength and righting response, hyporesponsivity to auditory stimuli, reduced tonus and marked loss of coordination with righting reflex. No significant concentration-dependent, macroscopic findings were noted at necropsy in animals at scheduled kill at 14 days, there was evidence of distended and congested lungs, lung oedema, pale appearance of some parenchymal organs and an increase in the level of foamy secretions in the gastrointestinal tract in animals that died prematurely.

The NOAEL could be not be identified as clinical signs of toxicity were seen in all dose groups in both sexes and because of the number of deaths in females in all dose groups. The LC_{50} was 0.572 mg/L air for male and female rats (Pauluhn, 1996).

In GLP-compliant pilot studies, mice (ICO-OF1, 4 males/group) and rats (Wistar; 4 males/group) were exposed to flumethrin (purity 94%) at 0, 2.5, 11.1 and 59.7 mg/m^3, using a 1 : 1 mixture of polyethylene glycol and ethanol as the vehicle, via directed-flow, nose-only exposure for 60 minutes. The animals were allowed to recover for 30–60 minutes and monitored for 1 week before scheduled kill. MMAD ranged from 1.08 to 1.14 μm and GSD from 1.89 to 1.92 μm, and 93% or more of the aerosol mass was less than 3 μm. Control groups included (1) conditioned air and (2) aerosolized vehicle.

At concentrations at or greater than 11.1 mg/m^3, the animals showed signs of laboured breathing, bradypnoea (rats), salivation (rats) and piloerection. At 59.7 mg/m^3, reduced motility, bradypnoea (mice), prostration and dyspnoea (rats) were noted. A concentration-dependent decrease in body-weight gain was noted in all treated rat groups but the decrease was smaller in mice, becoming significant only at 59.7 mg/m^3. A concentration-dependent decrease in respiratory rate and an increase in respiration minute volume and apnoea time were noted in both species. No concentration-dependent adverse gross pathological changes were noted. Exposure concentrations producing a 50% respiratory rate decrease (RD_{50}) were determined to be 48 mg/m^3 (in mice) and 19.4 mg/m^3 (in rats), based on a decrease in respiratory minute volume rather than respiratory rate (Pauluhn, 1997a,b).

Rats (Wistar; 10/sex per dose) were exposed to flumethrin (purity 94.4%) at 0, 0.12, 1.33 and 22.4 mg/m^3, using a 1 : 1 mixture of PEG400 and ethanol as

the vehicle, via directed-flow, nose-only exposure, for 6 hours/day, 5 days/week for 4 weeks. MMAD was approximately 1.1 μm, GSD approximately 2 μm, and more than 90% of the aerosol mass was 3 μm or less. An additional satellite group of four males received 22.4 mg/m^3 to assess cardiovascular parameters.

There were no deaths, and clinical signs were limited to piloerection and ungroomed coat in the 1.33 mg/m^3 group. Atony, bradypnoea and reduced motility were also observed at 22.4 mg/m^3. These effects were found to be worse with exposure (noting also that the intensity in general was greater in female than male rats) but resolved during the exposure-free weekends. Concentration-dependent decreases in body (rectal) temperature were noted at 1.33 and 22.4 mg/m^3, but the decrease in body weight in both males and females at the highest dose during treatment were abrogated by a marked increase in body-weight gain during the exposure-free weekends. No significant findings were noted in cardiovascular parameters, FOB, oxygen consumption, carbon dioxide production, haematology and clinical chemistry, urine analysis and ophthalmological examination. Hepatic monooxygenase activity was significantly reduced in males (aminopyrine-*N*-demethylase) and females (microsomal P450 content) at the two highest doses. There was an overall trend for reduced tidal volume at 1.33 and 22.4 mg/m^3 as well as an increase in the respiration rate and apnoea time in the high-dose group. A reduction in thymic and splenic weights at the high dose was considered treatment related, but there were no significant histopathological findings in these or other organs/tissues.

The overall systemic NOAEL was 0.12 mg/m^3 based on clinical signs and reduced tidal volume at 1.33 mg/m^3 (Pauluhn, 1997c).

2.2.3 Long-term studies of toxicity and carcinogenicity

(a) Mice

In a GLP-compliant carcinogenicity study, flumethrin (purity 93.7%) was administered in the diet to mice (Crl:CD-1 (ICR) BR; 50/sex per dose at all concentrations except 10/sex per dose at 1 mg/kg feed) at 0, 1, 3, 15 or 30 mg/kg feed for 79 weeks (18 months). These dietary values were equal to 0, 0.12, 0.39, 1.97 and 4.56 mg/kg bw per day for males and 0, 0.15, 0.52, 2.34 and 4.95 mg/kg bw per day for females, respectively. Animals were observed for clinical signs of toxicity, body weight and feed intake. Haematology was evaluated in a subset of animals at weeks 52/53 and 76. All animals that died prematurely or were killed at study termination underwent gross pathological and histopathological examinations. Some organs (brain, adrenals, liver, spleen, kidneys and testes) were weighed at necropsy. Urine analysis and assessment of clinical chemistry and ophthalmological measurements were not undertaken.

Clinical signs of toxicity at 1 and 3 mg/kg feed were limited to slight and transient red spots on the tails of both sexes. At 15 mg/kg feed, animals were observed to have auricle loss; wounds on hairless areas; reduced eye size (males; details not given in the study report); redness of the eyes (males); and high stepping gait (both sexes). Similar observations were made at 30 mg/kg feed in addition to piloerection and wounds on hairless areas (both sexes); inflammation; loss of hair and suppurative eyes (males); and deformed auricles (both sexes). Histopathological examination found that, in general, the incidence and severity of epidermal hyperplasia, inflammation and/or ulceration of affected skin was increased in both sexes at 15 and 30 mg/kg feed. Body weight was transiently but significantly reduced in males at 30 mg/kg feed. The incidence of unscheduled deaths in males during the study were 10, 3 (1), 8 (2), 13 and 25 (3), respectively, for the different dose groups (values in parentheses represent deaths related to blood sampling errors). In females, unscheduled deaths were 9, 3, 14, 11 and 19 (1), respectively. Intercurrent deaths were dose-dependently increased in males, becoming statistically significant at 30 mg/kg feed.

Body weight was transiently but significantly reduced in males at 30 mg/kg feed. Feed intake was comparable in all dose groups, except for a slight increase in males at 30 mg/kg feed. No significant dose-dependent changes in haematological parameters were noted.

There were no significant dose-related changes in organs weights at necropsy. Non-neoplastic changes included enlarged mandibular lymph nodes in males at 30 mg/kg feed (8/50); plasmocytosis in males at 15 mg/kg feed and males and females at 30 mg/kg feed; increased lymphoid hyperplasia in males at 15 and 30 mg/kg feed; enlarged or swollen spleen in males at 15 (3/50) and 30 mg/kg feed (6/50); slight increase in the incidence of diffuse glandular hyperplasia in stomach in females at 15 and 30 mg/kg feed; increased incidence of chronic nephropathy in males and females at 30 mg/kg feed; decreased hepatocellular glycogen content and increased hepatocellular atrophy in males at 15 mg/kg feed and males and females at 30 mg/kg feed; and increased thymic atrophy in both sexes at 30 mg/kg feed (overall incidence in males: 1, –, 2, 3 and 7; in females: 2, –, –, 1 and 4, at 0, 1, 3, 15 and 30 mg/kg feed, respectively). These effects were considered secondary to the skin changes and poor condition of the higher dose groups. There were no dose- or sex-related changes in the incidence of benign or malignant tumours or the total number of tumours (Table 3).

The NOAEL for non-neoplastic changes in this study was 3 mg/kg feed (equal to 0.39 mg/kg bw per day for males and 0.52 mg/kg bw per day for females) based on clinical signs and non-neoplastic histopathological findings at the next highest dose of 15 mg/kg feed (1.97 and 2.34 mg/kg bw per day for males and females, respectively) (Wirnitzer & Hartmann, 1999).

Table 3
Number of tumours in mice treated with flumethrin for 18 months

	No. of animals with the finding per sex and flumethrin concentration [a]									
	Males					Females				
	0 mg/kg feed	1 mg/kg feed	3 mg/kg feed	15 mg/ kg feed	30 mg/ kg feed	0 mg/kg feed	1 mg/kg feed	3 mg/kg feed	15 mg/ kg feed	30 mg/ kg feed
No. of mice examined	50	10	50	50	50	50	10	50	50	50
Tumours										
Benign	12	0	14	9	5	8	0	7	14	4
Malignant	14	3	10	13	7	14	3	16	20	8
Total	26	3	24	22	12	22	3	23	34	12

F: female; M: male; no.: number
[a] Some animals may have had both benign and malignant tumours.

(b) **Rats**

In a GLP-compliant combined chronic toxicity and carcinogenicity study, flumethrin (purity 95.8–93.17% during the course of the study) was administered in the diet to rats (Wistar, Hsd CpB:WU; 50/sex per dose) to give a dose of 0, 0.7, 2 or 4 (reduced from 6 at week 18) mg/kg bw per day for 106 weeks (2 years). Additional groups of animals (10/sex per dose) were given the same doses of flumethrin and were killed after 1 year of treatment. Animals were observed for clinical signs of toxicity and assessed for body weight, feed intake and water consumption. Other assessments included haematology, clinical chemistry including blood glucose and urine analysis. All animals that died prematurely or were killed at 1 or 2 years underwent gross pathological and histopathological examinations. Organs were weighed at necropsy. Due to severe skin changes (wounds), 10 males and 10 females receiving 6 mg/kg bw per day were euthanized in week 17 of treatment. The highest dose was subsequently reduced to 4 mg/kg bw per day from week 18 onwards for all surviving high-dose animals.

No clinical signs of toxicity were observed in the control group. At the low dose, clinical signs were limited to incidental skin changes in two males; this was not considered an adverse finding. Loss of hair (females) and skin changes (usually shortly after the start of treatment and with subsequent recovery) were noted in mid-dose males (10/60) and females (9/60). High-dose animals had piloerection and loss of hair, with skin changes in both males (42/60) and females (26/60). In addition to the 10 high-dose animals/sex that were euthanized in a moribund condition in week 17, of the animals scheduled for 2 years of treatment (50/sex per dose), 15, 17, 13 and 16 males and 14, 24, 14 and 15 females (at 0, 0.7, 2 and 4/6 mg/kg bw per day, respectively) died spontaneously during the study period or were euthanized in a moribund condition. Mortality in the high-dose

group was statistically significantly increased at 6 or 4 mg/kg bw per day. One mid-dose female (2 mg/kg bw per day) was euthanized (week 50) in moribund condition; pathological examination revealed a pituitary gland adenoma with distinct compression of the brain. This finding was not considered treatment related given its isolated incidence in this study and because it is considered a spontaneous tumour in rats.

Body weight was not significantly affected in males at up to 2 mg/kg bw per day and females at up to 4 mg/kg bw per day. Body weight was reduced in high-dose males, at a maximum of 12.9% compared with controls at week 14 and 7.7% compared with controls at the end of the study. There were no significant differences between treatment groups in feed and water intake. Urine analysis did not reveal any adverse findings. Similarly, there were no treatment-related opthalmological findings. Haematological analysis found a slight increase in anisocytosis and macrocytosis in high-dose females in week 105; increased haematopoietic activity in spleen in high-dose males and females; and evidence of fatty atrophy of the femoral bone marrow in high-dose females and sternal bone marrow in high-dose males and females. Clinical chemistry of animals at 4 mg/kg bw per day revealed a slight but significant increase in peripheral blood alanine aminotransferase in males (week 105: 42.2 versus 33.6 U/h in controls); a slight decrease in triglyceride levels in males and females in week 27; a slight decrease in protein levels in males in week 27 and 79; and a slight decrease in blood glucose levels in females in week 52 (3.79 versus 4.14 mmol/L in controls). However, these values were all within their respective historical control ranges.

Apart from a trend in the reduction of splenic weight with treatment, there were no significant treatment-related changes in organ weights. Necropsy and histopathological analysis at the interim kill indicated some evidence of sex organ atrophy (testes, epididymides, prostate and seminal vesicles) at high dose, but this was considered to be related to the poor condition of the animals and not a direct effect of flumethrin on the organs per se. At the study's conclusion, there was a trend in increased myodegeneration and sciatic nerve fibre degeneration and alveolar macrophages in males at 4 mg/kg bw per day. At the interim kill, there was an increase in the vacuolation of the adrenal cortex in males at 2 (6/10) and 4 mg/kg bw per day (5/10) compared with controls (2/10). Decreased hepatic glycogen content was observed in 9/10 males and 7/10 females at 4 mg/kg bw per day, and in 1/10 females at 2 mg/kg bw per day.

Apart from a significant reduction in par distalis adenomas in females at 2 mg/kg bw per day compared with the controls and a slight increase in the incidence of Leydig cell tumours (benign) in males (overall incidence: 0, 1, 1 and 3), there were no dose-related increases in benign or malignant tumours in either sex (Table 4).

Table 4
Number of tumours in rats treated with flumethrin for 2 years

	No. of animals with the finding per sex and flumethrin concentration							
	Males				Females			
	0 mg/kg feed	0.7 mg/kg feed	2 mg/kg feed	4 mg/kg feed	0 mg/kg feed	0.7 mg/kg feed	2 mg/kg feed	4 mg/kg feed
No. of rats examined	50	50	50	50	49	50	50	50
Tumours								
Benign [a,b]	40	31	36	26	54	56	49	49
Malignant [a,b]	6	5	7	3	9	12	5	11
Total	46	36	43	29	63	68	54	60

no.: number
[a] Bilateral/multiple tumours occurring at the same site and of the same type were classified as a single tumour.
[b] Some animals may have had both benign and malignant tumours.

The NOAEL was 0.7 mg/kg bw per day based on clinical findings at 2 mg/kg bw per day (Schladt & Deschl, 1999).

2.2.4 Genotoxicity

The genotoxic potential of flumethrin and its individual isomers has been examined in a number of in vitro and in vivo studies. Most studies were not conducted under GLP-compliant conditions, but were adequately documented (Table 5).

2.2.5 Reproductive and developmental toxicity

(a) Multigeneration reproductive toxicity

In a non-GLP-compliant pilot toxicity study in rats (Wistar (Crl:(WI) WU BR); 5/sex per dose), Eiben (2006a) administered flumethrin (purity 95%) by gavage, using a corn oil vehicle, at 0, 0.08, 0.4 or 2.0 mg/kg bw per day for 79 days and at 0, 3.0, 4.0 or 5.0 mg/kg bw per day on days 80–134. Animals were mated 28 days into the higher dosing regimen. All animals (parents and pups) were killed on day 134, which was equivalent to postnatal day 4. One female at 5.0 mg/kg bw per day was euthanized in a moribund state in week 17 due to a haematoma following blood sampling, and one control female with 12 fetuses died while littering in week 18. No clinical signs were noted in males and non-pregnant females at doses up to 5.0 mg/kg bw per day. In pregnant and lactating females administered either 4.0 or 5.0 mg/kg bw per day, salivation, piloerection, cyanosis, reduced motility, staggering gait, emaciation, inactivity and/or lying on their side were evident. No treatment-related effect on body weight was noted in either parental males or females at doses up to and including 2.0 mg/kg bw per day, and generally for

Table 5
Results of genotoxicity assays on flumethrin

Test system	Test object	Concentration / dose	Purity (%)	Results		Reference
				Without S9	With S9	
Flumethrin						
Reverse mutation	*Salmonella typhimurium* TA100, TA1535, TA1537, TA98	12 500 µg/mL [a]	83.3	Negative	Negative	Herbold (1981)
Reverse mutation	*S. typhimurium* TA100, TA1535, TA1537, TA98	15 625 µg/plate	97.6	Negative – inconclusive	Negative, inconclusive, weakly positive	Herbold (1984)
Reverse mutation	*S. typhimurium* TA100, TA1535, TA1537, TA98	5 000 µg/plate	94.6	Negative	Negative b	Gahlmann (1993a)
Reverse mutation	*S. typhimurium* TA100, TA1535, TA1537, TA98	15 000 µg/plate	94.6	Negative	Negative	Gahlmann (1993b)
Reverse mutation	*Saccharomyces cerevisiae* D7	10 000 µg/mL	92.7	Negative	Negative	Herbold (1985a)
Reverse mutation	*S. typhimurium* TA1535, TA100, TA1537, TA98, TA102	5 000 µg/plate [c]	95.8	Negative	Negative	Herbold (2006)
Point mutation (*tk* locus) assay	Mouse lymphoma L5178Y cells	1 000 µg/mL	92.7	Negative	Negative	Cifone & Myhr (1985)
Point mutation (*hprt* locus) assay	Chinese hamster lung V79 cells	100 µg/mL	95.1	Inconclusive	Negative	Brendler-Schwaab (1995)
Point mutation	Mouse lymphoma	1 200 µg/mL	86.6	Negative	Weakly positive	Cifone & Myhr (1986)
Unscheduled DNA synthesis	Rat hepatocyte primary culture	300 µg/mL	94.6	Negative	NA	Brendler-Schwaab (1994)
Chromosomal aberration	Chinese hamster lung V79 cells (18- & 30-h sampling)	125 µg/mL	95.1	Negative	Negative	Herbold (1995b)
Chromosomal aberration	Human lymphocyte primary culture (24-h sampling)	1 000 µg/mL	92.7	Negative	Negative	Herbold (1985b)
Chromosomal aberration	Chinese hamster lung V79 cells	192 µg/mL	95.8	Negative	Negative	Entian (2007)
Chromosomal aberration	Chinese hamster lung V79 cells (18- & 30-h sampling)	100 µg/mL	95.8	Negative	Negative	Thum (2007)
Micronucleus induction	Mouse bone marrow in vivo (24-, 48- & 72-h sampling)	50 mg/kg bw (PO)	93.5	Negative	NA	Herbold (1986)
Micronucleus induction	Mouse bone marrow in vivo (16-, 24- & 48-h sampling)	1 000 mg/kg (IP)	95.1	Negative	NA	Herbold (1995a)
Micronucleus induction	Mouse bone marrow	5 325 mg/kg bw (dermal); 128 mg/kg bw (IP)	60	Weak	NA	Nakano, Rabello-Gay & Pereira (1996)
Micronucleus induction	Mouse bone marrow	500 mg/kg bw (IP)	95.8	Negative	NA	Herbold (2007)
Flumethrin-*trans*-*Z*-1 isomer						
Reverse mutation	*S. typhimurium* TA98	15 000 µg/plate	98.3	Negative	Negative	Herbold (1990a)
Flumethrin-*trans*-*Z*-2 isomer						
Reverse mutation	*S. typhimurium* TA98	15 000 µg/plate	95.4	Negative	Negative	Herbold (1990b)

bw: body weight; hprt: hypoxanthine–guanine phosphoribosyltransferase; IP: intraperitoneal; NA: not applicable; PO: by mouth; S9: 9000 × *g* supernatant fraction from rat liver homogenate

[a] Precipitation observed at ≥2500 µg/plate. No cytotoxicity at ≤8000 µg/plate.

[b] An additional test in the presence of 30% S9; all bacterial studies include duplicate experiments with 10% S9.

[c] Precipitation observed at 5000 µg/plate; weakly cytotoxic at 5000 µg/plate.

males up to 3.0 mg/kg bw per day; at higher doses, reduced body-weight gain was observed. Feed and water intake were unaffected by treatment.

Mean pup weight at birth and on postnatal day 4 were reduced at 3.0 mg/kg bw per day and above and the number of litters surviving up to postnatal day 4 was reduced at 4.0 mg/kg bw per day and above; however, the numbers of pups and litters in this study were small from the outset. There were no significant findings at necropsy in the parental animals (F_1 pups were not assessed). Increased absolute adrenal weights were noted in females treated at 4.0 and 5.0 mg/kg bw per day.

The NOAEL for parental toxicity was 3.0 mg/kg bw per day for males and 2.0 mg/kg bw per day for females based on effects on body weight and clinical signs at the next highest dose.

The NOAEL for offspring toxicity was 2.0 mg/kg bw per day based on a reduced pup weight at 3.0 mg/kg bw per day.

Due to the small sample size, a NOAEL for reproductive toxicity could not be identified in this study.

(See the Krebber, 2006b, study in section 2.1.1(a) for toxicokinetic analysis of flumethrin reported in this study.)

In a two-generation, GLP-compliant reproductive toxicity study, rats (Wistar/HAN; 30/sex per group) were fed diets containing flumethrin (45.6% in Aerosil 200 [fumed silica]) at concentrations of 0, 1, 5 or 50 mg/kg feed (equal to 0, 0.07, 0.37 and 3.78 mg/kg bw per day for F_0 males and 0, 0.08, 0.40 and 4.05 mg/kg bw per day for F_0 females). F_0 animals received the test diet for 84 days before mating and then during the mating period, gestation and lactation until postpartum day 21. The F_1 parental animals received the test diet from the age of 4–7 weeks, for 105 days before mating and thereafter over the same period as the F_0 animals.

No effects of treatment were discernible at either 1 or 5 mg/kg feed in any generation. After treatment with 50 mg/kg feed, skin lesions developed on F_0 males and females and F_1 females. F_0 males at 50 mg/kg feed had reduced feed consumption before mating and F_1 males had reduced feed consumption in all phases; F_0 females had a reduced feed consumption in all phases and F_1 females had a reduced feed consumption during the two lactation phases. The body-weight development of F_0 males and F_1 males and females was retarded in all phases. The reductions in feed consumption and body-weight gain were seen as early as the first week of dosing before mating.

Survival in all F_1 and F_2 animals at 50 mg/kg feed was lower during the first 4 days after delivery, but the losses up to day 21 after delivery were greatest in F_{1b}, F_{2a} and F_{2b} animals. In addition, the weight gain of F_1 and F_2 pups were retarded. A higher incidence of pups with cramped or hunched posture, stiff limbs in caudal posture and/or pectus carinatum (pigeon chest) was seen, with

hypothermia; vocalization was also more frequent. These observations were probably secondary to the toxic effects on the parents. No malformations were found. Investigations of the haematological status of the F_1 parental animals gave no indication of treatment-related changes.

The NOAEL for parental toxicity was 5 mg/kg feed (equal to 0.37 mg/kg bw per day for males and 0.40 mg/kg bw per day for females) based on skin lesions at 50 mg/kg feed.

The NOAEL for offspring toxicity was 5 mg/kg feed (equal to 0.37 mg/kg bw per day for males and 0.40 mg/kg bw per day for females) based on reduced pup survival and retarded body-weight gain at 50 mg/kg feed.

The NOAEL for reproductive toxicity was 50 mg/kg feed (equal to 3.78 mg/kg bw per day for males and 4.05 mg/kg bw per day for females), the highest dose tested (Dotti et al., 1992).

In a two-generation, GLP-compliant reproductive toxicity study, rats (Wistar (Crl:(WI) Wu BR)); 25/sex per dose; 5 weeks old at study start) were administered flumethrin (purity 95.8%) via gavage, using a corn oil vehicle, at doses of 0, 0.5, 1 or 3 mg/kg bw per day. The F_0 rats were dosed for 10 weeks before mating and during cohabitation and mating. At the end of the mating period, F_0 males were killed while F_0 females continued to receive treatment until scheduled kill at postnatal day 28. F_1 pups were culled to 4/sex per litter at postnatal day 4 and reared to postnatal day 28. F_1 pups (25/sex per dose) selected for subsequent mating were treated for an additional 10 weeks prior to cohabitation and mating. All F_1 parents and F_2 pups were killed at the F_2 pups' postnatal day 28.

Unscheduled deaths were unrelated to the test substance (one F_0 male at 0.5 mg/kg bw per day euthanized on day 25 due to gavage error; one F_0 female at 3 mg/kg bw per day euthanized moribund on day 67 due to blood sampling error; one F_1 female at 1 mg/kg bw per day euthanized due to difficult parturition on gestation day 22). Clinical signs were generally unremarkable and limited to a small number of high-dose animals with loss of hair and alopecia in F_0 animals. For all rats, there were no treatment-related effects on body weight at doses up to and including 1 mg/kg. At 3 mg/kg bw per day, F_0 and F_1 males and F_1 females had significantly lower body weights than the controls. At this dose, F_0 and F_1 dams had a reduced feed intake.

There were no significant treatment-related effects on sperm (assessed only in animals at 0 and 3 mg/kg bw per day) or measures of reproductive function. The rearing index was slightly, though not statistically significantly, reduced at 3 mg/kg bw per day in F_0 (75.0% versus 95.8% in controls) and F_1 (87.5% versus 95.2% in controls). No effect on gestation length was evident. Statistically significant organ weight changes at necropsy were limited to lower absolute and relative liver weights (by ≤10%) in F_0 and F_1 animals at 1 and 3 mg/kg bw per day. Other organ weight changes were considered incidental to treatment.

No significant macroscopic findings were noted in the F_0 and F_1 animals up to and including 3 mg/kg bw per day. Furthermore, no histopathological lesions were apparent up to and including 3 mg/kg bw per day. No treatment-related effects were identified on F_1 and F_2 birth indices, mean litter size at birth, sex distribution, number of pups born, implantation sites or effects on lactation. However, the viability index was reduced in high-dose F_1 (63% versus 96% in controls) and F_2 (77% versus 94% in controls) pups due to increased deaths in pups on postnatal days 0–4 at the high dose.

Significant clinical findings in pups were limited to high-dose F_1 and F_2 pups; the findings included a reduction in number of pups and litters, with no milk spots and thin, relatively small appearance. These findings were concordant with significantly reduced mean pup weight throughout the lactation period in F_1 pups (males: 71.2 versus 80.0 g; females: 65.9 versus 74.0 g in controls at lactation day 28) and F_2 (males: 69.4 versus 79.5 g; females: 64.2 versus 73.6 g in controls at lactation day 28). Sexual maturation in F_1 pups was slightly delayed with a statistically significant delay in the time to vaginal opening in high-dose females (35.6 versus 33.8 days in controls). Belanopreputial separation was unaffected. In F_2 pups, there was no effect on anogenital distance (only measure of maturation undertaken in this generation). There were no biologically significant organ weight changes in the F_1 or F_2 weanlings. Relative brain weight was significantly increased in F_2 pups in both sexes (+11%) at the high dose. Relative thymus weight was also significantly increased in both males (521 versus 443 mg/100 g body weight) and females (537 versus 456 mg/100 g body weight) in high-dose F_2 animals. The change in females was slightly outside the range of historical control data (443–526 mg/100 g body) for the testing laboratory. (See section 2.1.1(a) for toxicokinetic data from F_0 animals in this study.)

The NOAEL for parental toxicity was 1 mg/kg bw per day based on reduced body weight at 3 mg/kg bw per day in all generations.

The NOAEL for offspring toxicity was 1 mg/kg bw based on reduced pup weight, reduced pup viability and clinical findings in pups at 3 mg/kg bw per day.

The NOAEL for reproductive toxicity was 3 mg/kg bw per day, the highest dose tested (Eiben, 2008).

(b) Developmental toxicity

(i) Rats

Mated female rats (Crl:CD Br rats; 28/dose) were administered flumethrin (purity 93.5%) by gavage at 0, 0.5, 1.0 or 2.0 mg/kg bw per day on gestation days 6–15 and killed on gestation day 20. Doses were formulated as a 0.4 mg/mL solution in distilled water containing 5% Emulphor EL-719 (ethoxylated castor oil) and 5% ethanol.

There were no unscheduled deaths in the dams. No significant clinical signs were evident in either control or low-dose dams. At 1.0 mg/kg bw per day, some dams exhibited salivation (7/28, 25%) from the first day of treatment. Salivation was also observed on the first day of treatment in all dams at 2.0 mg/kg bw per day; hypoactivity, ptosis, ataxia, lachrymation and urine-stained ventral surface were also evident. High-dose dams also had reduced mean feed consumption and mean body weight and body-weight gain. Fetotoxicity was evident at 2.0 mg/kg bw per day by a slight but significant reduction in median placental weights (0.48 versus 0.53 g in controls) and median combined fetal weights (3.4 versus 3.8 g in controls); and an increased incidence of fetuses with reduced ossification at several sites, notably the skull bones (67% versus 42% in controls) and cervical vertebral arches (16% versus 1% in controls).

The NOAEL for maternal toxicity was 0.5 mg/kg bw per day based on clinical signs at 1.0 mg/kg bw per day.

The NOAEL for embryo/fetal toxicity was 1.0 mg/kg bw per day based on reduced placental weight, reduced fetal weights and an increase in the incidence of skeletal variations at 2.0 mg/kg bw per day.

There was no evidence of teratogenicity in this study (Kowalski, Clemens & Hartnagel, 1987).

Mated female rats (Wistar (Hsd CpB:W); 27/dose) were administered flumethrin (purity 94.0%) via gavage, using a corn oil vehicle, at 0, 0.75, 2 or 5 mg/kg bw per day on gestation days 6–19 and killed on gestation day 20.

There were no unscheduled deaths. No significant clinical signs were noted at 0.75 mg/kg bw per day, but salivation was evident at both 2 mg/kg bw per day (6/27) and 5 mg/kg bw per day (27/27). The high-dose group had small wounds about the ears, but these were associated with animals scratching out their ear tags. Hypothermia and an additional wound at the neck were also reported in one animal each at the high dose. Feed intake was slightly reduced at the mid dose; this was more evident at the high dose. Approximately one third of the high-dose animals had a reduced water intake. Body weight was unaffected at the low dose, and while statistically significant at the mid dose, was within the historical control range. On the other hand, body-weight loss was noted in the high-dose animals on gestation days 6–7 and reduced body-weight gain was noted during the rest of the treatment and gestation period.

There were no significant dose-dependent changes in pre- and postimplantation losses. Placental weight was significantly lower in high-dose animals compared with controls (0.47 versus 0.61 g). Fetal weights were also significantly reduced compared to controls (2.79 versus 3.62 g) and were below the historical control range. The incidence of necrotic placental borders was higher in the mid- and high-dose animals, but not when considered on a litter basis. There were no significant external or visceral changes in fetuses. Although

not statistically significant, there was a small increase in the dysplasia of forelimb bones (6 fetuses in 3 litters versus 1 fetus in 1 litter) and microphthalmia (8 fetuses in 5 litters versus 4 fetuses in 4 litters), but these incidences generally fell within historical control ranges. Delays in skeletal ossification and increased variations, noted predominantly at the high dose, were not considered toxicologically significant as they were concordant with delayed development from reduced fetal weights.

The NOAEL for maternal toxicity was 0.75 mg/kg bw per day based on salivation at 2 mg/kg bw per day.

The NOAEL for embryo/fetal toxicity was 2 mg/kg/bw per day based on reduced fetal weight at 5 mg/kg bw per day (Klaus, 1999).

(ii) Rabbits

Artificially inseminated rabbits (American Dutch; 17/dose) were administered flumethrin (purity 93.5%) by gavage at 0, 0.5, 1.7 or 6.0 mg/kg bw per day on gestation days 7–19. Doses were formulated as a 0.4 mg/mL solution in distilled water containing 5% Emulphor EL-719 (ethoxylated castor oil) and 5% ethanol. The does were killed on gestation day 28.

There were no unscheduled deaths. Four does aborted (low dose, day 23; low dose, day 18; high dose, day 20; high dose, day 21), but these were not considered treatment related. There were no significant clinical signs in any of the treated does. While no adverse effects were noted in animals receiving either 0.5 or 1.7 mg/kg bw per day, high-dose animals had reduced feed intake and reduced body weights during the treatment period, but body weights were comparable to controls after cessation of treatment. Fetal mortality was not affected by treatment (4 control [3/4 from one litter]; 0 low dose; 1 mid dose; and 6 high dose [5/6 from one litter]. At the high dose, there was a nonstatistically significant trend in reduced fetal body weight (33.9 versus 35.9 g in controls) that was lower than the testing laboratory historical control range (34.7–39.8 g). There were no statistically or toxicologically significant changes in incidence of skeletal, external and viscera changes or malformations/variations in the fetuses.

The NOAEL for maternal toxicity was 1.7 mg/kg bw per day based on reduced feed intake and body weight at 6.0 mg/kg bw per day.

The NOAEL for embryo/fetal toxicity was 1.7 mg/kg bw per day based on reduced fetal body weight at 6.0 mg/kg bw per day.

There was no evidence of teratogenicity in this study (Clemens & Hartnagel, 1987).

Mated female rabbits (Himalayan (CHBB:HM); 22/dose) were administered flumethrin (purity 95.8%) via gavage at 0, 0.5, 1.5 or 4.5 mg/kg bw per day on gestation days 6–8 and killed on gestation day 29.

There were no unscheduled deaths. Four high-dose does aborted (on gestation days 25, 27, 27 and 29). These animals had increased incidence of encrusted wounds at the throat or forelimbs, cold ears, soft and/or coloured faeces, discoloured urine and/or whitish or reddish excretions. For only 1 day each, two of the females had a tilted head. Limb swelling was apparent at both 1.5 and 4.5 mg/kg per day. Severely reduced to no feed intake and marked to severe body-weight loss were noted in the high-dose does that aborted.

The high-dose group had a reduced feed intake on gestation days 6–26; on occasion there was no feed intake. The high-dose group also had a reduced water intake, which could account for the discoloured urine. Body-weight gain in the high-dose group (37.6 g) was much lower than in the control group (248.1 g) on gestation days 0–29. At necropsy, six of the high-dose does, including those that aborted, had abdominal findings (hardened fatty tissue, enlarged gall bladder and small intestine filled with light green fluid). An enlarged gall bladder was also noted in one control and one 1.5 mg/kg per day doe, but the incidence in these animals was within the historical control range. The number of corpora lutea and the preimplantation loss in the high-dose group were marginally but not statistically significantly increased. The gestation rate in the high-dose group was reduced, but this was related to the four abortions. In the high-dose group, placenta weight was slightly but not significantly lower (3.94 versus 4.05 g in controls); postimplantation loss was higher (means: 1.7/female versus 0.3/female in controls); and fetal body weight was lower (32.5 versus 36.1 g in controls). High-dose does had an increased incidence of coarsely grained placentas (12% versus 0% in controls).

There was an increase in the incidence of liver lobulations in high-dose fetuses, but this finding was limited to one litter only from a doe with severe maternotoxicity. These fetuses also had a very low body weight at necropsy (22.7 g). Incidental findings of retinal folding were within the historical control range and may have been a fixation artefact. There was a statistically significant increase in the incidence of fused sternebrae at 1.5 mg/kg bw per day, when assessed on a fetal basis (12.6% affected fetuses compared with the upper range of historical controls of 11.9%), but not on a litter basis (45.5% compared with the upper range of 47.1%). At the high dose, there was a statistically significant retarded ossification at a few localizations that were outside of the historical control range, but were not significant on a litter basis. The incidence of fused sternebrae was statistically significantly increased in the high-dose group on a fetal basis (17.9% versus 3.8% in controls), but not on a litter basis (47.1% versus 25% in controls), although this was at the upper end of the historical control range (47.1%) for this strain of rabbit.

The NOAEL for maternal toxicity was 0.5 mg/kg bw per day based on clinical signs at 1.5 mg/kg bw per day.

The NOAEL for embryo/fetal toxicity was 1.5 mg/kg bw per day based on an increase in skeletal variations in pups at 4.5 mg/kg bw per day (Langewische, 2009).

2.2.6 Special studies

(a) Immunotoxicity

The potential immunotoxicity of flumethrin (purity 96.0%) was assessed in a GLP-compliant, 4-week dietary study in rats (Wistar; 8/sex per dose) given flumethrin at 0, 10, 40 or 160 mg/kg feed (equal to 0, 0.8, 3.0 and 11.7 mg/kg bw per day for males and 0, 1.0, 3.5 and 12.3 mg/kg bw per day for females, respectively). There were no significant, treatment-related changes in body weight or splenic and thymic weights. There were no treatment-related effects on splenic cell counts, composition of splenic subpopulations (CD3, CD4+, CD8+, CD4+/CD8+, CD161+ (NK) and CD45RA+ (PANB), antibody titre (immunoglobulins A, G and M) in the animal sera or splenic plaque-forming cells.

The NOAEL for immunotoxicity was 160 mg/kg feed (equal to 11.7 and 12.3 mg/kg bw in males and females, respectively), the highest dose tested (Schladt & Vohr, 2009).

(b) Neurotoxicity

Acute oral neurotoxic effects were assessed in a GLP-compliant study in rats (Wistar; 12/sex per dose) given flumethrin (purity 96.0%), via gavage, using a corn oil vehicle, at 0, 1.12, 5.15 or 15.5 mg/kg bw, and subsequently, in male rats only (12/dose) at 0, 0.27 or 0.53 mg/kg bw. Animals were assessed for up to 14 days following dosing and evaluated for neurobehavioural and neuropathological changes. FOB and motor activity assessments were undertaken 1 week prior to treatment and 5 hours, 7 days and 14 days after treatment in the initial study, and 1 week prior to treatment and 5 hours after treatment in the subsequent study. Motor and locomotor activity were examined during each of the six 10-minute intervals following completion of the FOB in each animal at each assessment time point.

No adverse effects were observed at 0.27 or 0.53 mg/kg bw. At 1.12 mg/kg bw, nonstatistically significant reductions in motor and locomotor activity in males were noted on day 0; these resolved by day 7. At 5.15 mg/kg bw, the study report noted a statistically significant reduction in motor and locomotor activity in both sexes on day 0, which also resolved by day 7. Similar statistically significant effects were noted in both sexes at 15.5 mg/kg bw; however, these effects were not confirmed at each assessment time and no significant changes were observed in FOB at doses less than 15.5 mg/kg bw. There was a decreased incidence of animals standing normally or rearing, decreased activity/arousal,

decreased body temperature and an increased incidence of animals that did not respond to the approach response test at 15.5 mg/kg bw. In addition, animals at 15.5 mg/kg bw were also observed with salivation, staining and red-stained forepaws. There were no deaths or significant treatment-related effects on body or brain weight, or neurohistopathological findings.

The NOAEL was 1.12 mg/kg bw based on reduced motor and locomotor activity at 5.15 mg/kg bw and higher doses (Gilmore, 2008a).

In a GLP-compliant study, rats (Wistar; 20/sex per dose) were administered flumethrin (purity 92.7%), in an acetone : peanut oil (1 : 10) vehicle, by gavage at 0 or 20 mg/kg bw per day (males) and 0 or 10 mg/kg bw per day (females) for 4 days. Due to unscheduled deaths, doses were reduced to 10 and 5 mg/kg bw per day for males and females, respectively, and administration continued for another 10 days prior to a 31-day recovery period.

One male rat in the treatment group died on each of day 4, 5 and 7. There were no deaths in the female groups. Necropsy of these animals revealed distended lung with spotted parts; dark liver; pale spleen and kidney; and occasional ulcerous foci in the small intestine. During treatment, animals in general demonstrated apathy, reduced motility, accelerated breathing, salivation, twitching of the head and spastic gait, which subsequently gave rise to digging and shaking movements. Three to 4 days after doses were halved, the intensity of these clinical signs was reduced. Body-weight loss was marked in both sexes during the first 4 days of treatment, but body weight increased during the subsequent treatment at the lower dose and during the recovery period. Body weight normalized during the recovery period to match that of control animals. On cessation of treatment, animals displayed slight apathy and reduced motility for the first 2 days but no other significant effects after this time. There were no significant gross pathological or histopathological changes of note in peripheral or central nervous systems at the end of the recovery period.

A NOAEL was not identified, as effects were observed at the highest dose tested (Flucke & Schilde, 1988).

In a GLP-compliant study, rats (Wistar; 12/sex per dose) were administered flumethrin (purity 95.8%) by gavage, using corn oil as a vehicle, at 0, 1, 2.6 or 5.1 mg/kg bw per day for 13 weeks. Due to signs of toxicity, including death, the 10 mg/kg bw per day dose was reduced to 2.6 mg/kg bw per day from day 3 of treatment. One female rat at 10 mg/kg bw per day died on day 3 (salivation, nasal discharge and ventral wet staining); one female rat at 5.1 mg/kg bw per day was found to have ventral wet staining and was euthanized on day 31; two male rats at 5.1 mg/kg bw per day were euthanized due principally to digit removal and various staining and nasal discharge, on each of day 9 and 38, respectively; and one male rat at 5.1 mg/kg bw per day was euthanized in week 7 when it bit off and swallowed the gavage tube.

Clinical signs in control and low-dose animals were limited to occasional red nasal staining, which was considered not substance-related. At 2.6 and 5.1 mg/kg bw per day, there was a dose-dependent increase in the number of animals with urine, oral, lacrimal and perianal staining. At 5.1 mg/kg bw per day, salivation and red-staining of forepaws was also observed. Those animals that received 10 mg/kg bw per day for 3 days also demonstrated decreased activity and ataxia. Body-weight gain was statistically significantly reduced in male rats at 2.6 and 5.1 mg/kg bw per day compared with controls. A similar but nonstatistically significant trend was seen in high-dose females, with an increase in females at low and mid dose. Feed intake was significantly reduced in both males and females at 10 mg/kg bw per day, in females at 5.1 mg/kg bw per day in the first week and in males at 5.1 mg/kg bw per day during the entire treatment period.

No significant treatment-related changes were observed in FOB, motor activity or ophthalmic investigation. No treatment-related changes were noted in gross pathology (other than those animals that died or were euthanized) or in histopathological examination of neural and non-neural tissues from high-dose animals (the only group assessed).

The NOAEL was 1 mg/kg bw per day based on clinical signs observed at 2.6 mg/kg bw per day (Gilmore, 2008b).

In a GLP-compliant developmental neurotoxicity study, Wistar dams were administered flumethrin (purity 96.0%) by gavage at 0, 0.51, 0.97 or 1.90 mg/kg bw per day from gestation day 6 through to lactation day 21 (except gestation day 21, parturition). The vehicle was corn oil. Pups were culled on postnatal day 4 to 4/sex per litter, and weaned on postnatal day 21. Pups were allocated to four assessment groups, A–D. One male and/or one female per litter to give approximately 16 (minimum 10)/sex per dose level, representing at least 20 litters/group were assessed for (A) motor activity; (B) auditory startle; and (C) passive avoidance, water maze behaviour and FOB. On postnatal day 21, the whole brain was collected from animals (10/sex per group, representing 20 litters/group) for (D) micropathology and morphometric analysis, and at study termination (postnatal day 71). At approximately 50–60 days of age, randomly selected animals from sets A, B and C (a minimum of 10/sex per dose (at least 20 litters/group) underwent ophthalmological examination. At study termination (on ~ postnatal day 75), the brains from randomly selected animals from sets A, B and C (a minimum of 10/sex per dose; at least 20 litters/group) were collected and weighed.

In maternal animals, no significant treatment-related effects were noted at the low or mid dose. At the high dose, feed consumption was reduced on gestation days 13–20 and body-weight gain was reduced by 9% from gestation day 0 to 20. There were no treatment-related deaths, clinical signs during gestation and lactation periods, or findings noted in the FOB.

In F_1 pups, there were no significant treatment-related effects on litter parameters, pup viability, clinical signs during lactation or post weaning, developmental landmarks (sexual maturation), FOB, motor and locomotor activity, auditory startle, passive avoidance, water maze activity or ophthalmology. Body weight was significantly reduced in high-dose males (8%) and high-dose females (6%) on postnatal day 11, but only in high-dose males (8%) on postnatal day 17. Similarly, body-weight gain was significantly reduced in high-dose males (9%) and high-dose females (8%) on postnatal days 4–11, but only in high-dose males (9%) on postnatal days 4–17. Post-weaning body-weight differences were within historical control ranges for the treatment groups. There were no significant treatment-related effects noted on interim (postnatal day 21) or terminal organ (brain) and body weights, and no notable microscopic lesions.

The NOAEL for maternal toxicity was 0.97 mg/kg bw per day based on reduced body-weight gain at 1.90 mg/kg bw per day.

The NOAEL for offspring toxicity was 0.97 mg/kg bw per day based on body weight and body-weight changes in both males and females at 1.90 mg/kg bw per day.

The NOAEL for developmental neurotoxicity was 1.90 mg/kg bw per day, the highest dose tested (Sheets, Gilmore & Hose, 2008).

(c) Safety pharmacology

In a non-GLP-compliant study, rats (male Bor:WISW) and mice (Bor:CF1) were given flumethrin (purity not stated) by gavage, using PEG400 as vehicle, in a single oral dose of 0, 10, 31. 5 or 100 mg/kg bw.

No muscle-relaxant, analgesic, anticonvulsant or cataleptic effects were noted in either species. There was no evidence of impairment of central coordination, function, reflexes or neurotransmission in rats. In mice, flumethrin caused moderate stimulation of spontaneous motor activity (motility), which was statistically significant at 31.5 and 100 mg/kg bw. Orientation activity was also inhibited in these animals. Mice at the high dose showed a slight potentiation of the duration and depth of hexobarbital-induced anaesthesia (Starke, 1985).

The diuretic activity of flumethrin (purity not stated) in rats (Bor:WISW) was assessed after the gavage administration of single doses at 0, 10, 31.5 or 100 mg/kg bw, using PEG400 as the vehicle. Urine was collected over 6 hours, and sodium and potassium concentrations determined.

Clinical signs included increased salivation and reduced motor activity in a few treated animals. Treatment with flumethrin had no effect on urinary volume or sodium concentration. Potassium excretion was significantly increased at the 10 and 100 mg/kg bw doses but not the 31.5 mg/kg bw dose (Hirth, 1985). In light of the absence of a dose–response relationship and the relative large variability

between animals (coefficient of various ~30%), the toxicological significance of this observation is questionable.

The effect of flumethrin (purity not stated) on blood glucose and serum triglyceride levels was assessed in fed or unfed male Wistar (Han:BOE) rats. Animals (6/dose) were administered flumethrin by gavage at 0, 10, 31.5 or 100 mg/kg bw in a PEG400 vehicle. Blood glucose and triglyceride concentrations were measured at 30, 60, 120 and 240 minutes after dosing.

Blood glucose concentrations in fed animals at all doses and of fasted animals at 10 and 100 mg/kg bw were slightly (24–64%) but significantly increased for 60–240 minutes. As previously noted by JMPR, "as marked variation in the blood glucose concentrations are seen even under physiological conditions, these increases are not considered to be of particular relevance" (FAO/WHO, 1996). There was no effect on the triglyceride concentrations (Puls & Bischoff, 1985).

The effects of flumethrin on haemodynamics and cardiac contractility were assessed in dogs. Anaesthetized (chloralose–urethane–morphine) dogs (mongrel; 3/dose; mixed sexes) were administered flumethrin (purity 93.8%) at doses of 10, 31.5 or 100 mg/kg bw by gavage in a PEG400 vehicle and assessed up to 180 minutes after dosing.

Slight increases in heart rate, unrelated to dose, were observed in 1/3 animals given 10 and 100 mg/kg bw. However, these increases were not considered to be related to treatment (Knorr, 1986).

The effects of flumethrin on intestinal transit time, gastric tolerability and basal gastric acid secretion were assessed in rats (SD; male or female rats – only one sex was used for each test). Single oral doses of flumethrin (purity not stated) at 0, 10, 30 or 100 mg/kg bw were given to fasted animals by gavage in DHP-placebo (not defined) for tests of intestinal transit time and in 1% tylose for the other tests.

At the highest dose, a statistically significant increase in intestinal transit time (36%) was noted in a charcoal propulsion test. No gastric lesions were observed at autopsy, but excessive salivation, diarrhoea and hyperaemic stomach walls were noted. Intraduodenal administration of 10, 30 or 100 mg/kg bw to ethyl urethane–anaesthetized rats caused a slight, nonsignificant and non-dose-related reduction in acid secretion in the in situ perfused stomach (Bonabello & Grassi, 1987).

Flumethrin (10^{-9} to 10^{-5} g/mL) had no effect on antigen- or non-antigen-induced release of histamine from isolated rat peritoneal mast cells and had no direct effect on leukotriene D_4- or histamine-induced contractility in isolated guinea-pig tracheas (Gardiner, Hammond & Francis, 1985).

Single oral doses of flumethrin (purity not stated) in a PEG400 vehicle were administered to male rats (Bor:WISW; 5/dose) at 0, 10, 31.5 or 100 mg/kg

bw. Blood samples were taken 90 minutes later to investigate possible effects on blood.

No effects on coagulation, platelet aggregation or fibrinolytic were noted (Seuter & Perzborn, 1985).

2.3 Microbiological effects

Considering the chemical structure and mode of action of flumethrin, the Committee did not anticipate any adverse effects of flumethrin residues on human gastrointestinal microbiota.

2.4 Observations in humans

There was limited information on the effects of flumethrin on humans.

Box & Lee (1996) describe a case study where a 47-year-old farmer became ill after dipping sheep for 16 hours over the course of 2 days with a product containing flumethrine [*sic*]. Briefly, the individual reported an itchy rash that started at his elbows and forearms 24 hours after exposure, and spread over his whole body during the following 8 hours. Over the 2 days, symptoms worsened and he developed abdominal pain and vomiting associated with malaise and generalized muscle aches. Acute polyarthralgia developed 2 days later, initially affecting his hands and feet and then spreading to his knees, shoulders and elbows. He was in considerable pain and was admitted to hospital. The patient was febrile (39.1 °C); had widespread polyarthritis affecting metacarpophalangeal and proximal interphalangeal joints, wrists, elbows, shoulders and knees; and had extensive urticarial rash over his body. The key findings from clinical chemistry and haematological investigations revealed altered erythrocyte sedimentation rates (54 mm/hour) and C-reactive protein (26 mg/dL compared with the normal range of <1 mg/dL). Initial treatment included oral ibuprofen, chlorpheniramine and intramuscular pethidine, and antibiotics that were discontinued after negative screening for infection. Over the next 5 days, the rash became less itchy and arthralgia improved. No skin parathesiae was observed. On clinical review 6 weeks later, erythrocyte sedimentation rate and C-reactive proteins levels had returned to normal, and the farmer's symptoms had fully settled and he had returned to work. No blood or urine samples were stored and therefore no data were available on flumethrin concentrations in these matrices (Box & Lee, 1996).

While there is limited information on human exposure to and poisoning with flumethrin, a number of clinical case reports and reviews of pyrethroid poisoning from accidental, occupational and intentional exposure have been published (He et al., 1989; Bradberry et al., 2005).

Bradberry and colleagues noted that despite their worldwide use, there were relatively few reports of human pyrethroid poisonings and, in particular, few deaths following ingestion or occupational exposure. Occupational pyrethroid exposure is mainly through the skin, although inhalational exposure may occur with use in confined spaces. The main adverse effect from dermal exposure to pyrethroids is skin paraethesiae resulting from hyperactivity of cutaneous sensory nerve fibres. Paraesthesiae usually resolves in 12–24 hours and specific treatment is generally not required. Adverse clinical effects from pyrethroid ingestion can be present within minutes – sore throat, nausea, vomiting and abdominal pain. There may also be mouth ulceration, increased secretions and/or dysphagia – although these effects may also be contributed by co-formulants in the pyrethroid-containing pesticides. Systemic effects occur 24–48 hours after exposure – dizziness, headache and fatigue are common, whereas palpitations, chest tightness and blurred vision are less frequent. Coma and convulsion may also develop. Most patients recovered within 6 days with supportive management (Bradberry et al., 2005).

3. Comments

3.1 Biochemical data

The pharmacokinetics of flumethrin were studied in rats, rabbits, dogs, cattle and sheep. A number of investigations were performed as part of toxicological studies.

Flumethrin was rapidly but incompletely absorbed after oral administration in all species investigated. After a single oral dose of [F-phenyl-U-^{14}C]flumethrin, approximately 30% of the administered radioactivity was absorbed in rats (Steinke, Weber & Suwelack, 1983). Conversely, when rats were dosed with [Cl-phenyl-U-^{14}C]flumethrin, approximately 75% of the administered radioactivity was absorbed (Klein, 1993a), highlighting the importance of the position of the radiolabel. Plasma levels of radioactivity reached a maximum by 8 hours, but the elimination half-life ranged from 130 to 160 hours. Renal clearance was negligible (<1.2 mL/kg bw per hour). The concentration of radiolabel in organs was 3- to 50-fold lower than in plasma. Radiolabel accumulated in the plasma after multiple dosing, with 9–20% of the administered radioactivity still present in the body 7 days after administration (Klein, 1993a). The highest intensity of radioactivity was in the liver and the lowest in the CNS (Klein, 1993b).

Toxicokinetic data from a two-generation reproductive toxicity study in F_0 rats at days 38/39 in the premating period showed that, at steady state, C_{max} and

AUC of flumethrin were dose dependent but not dose proportional above 1 mg/kg bw per day. T_{max} was 4 hours (Hafner & Mueller, 2007; Krebber & Hoffend, 2007). In rabbits, the T_{max} after a single oral dose of 10 mg/kg bw was 5.4 hours, but the elimination half-life was 43.3 hours (Başçi & Eraslan, 2014).

Topical administration of flumethrin in dogs, a lactating cow and sheep indicated very limited dermal absorption in these species, with plasma levels (parent or radiolabel) generally less than the LOQ (Cameron & Phillips, 1986; Speirs & Donachie, 1999; Jons & Krebber, 2000). In the lactating cow, the highest levels of detectable radioactivity were recovered from the application site and underlying skin, followed by the urine, bile, kidney and liver. Radioactivity was also found in milk.

The primary metabolic pathway involves hydrolysis of the central ester bond to form flumethrin acid and 3-phenoxy-4-fluorobenzyl alcohol. Flumethrin acid accounted for 20–30% of the radiolabel recovered from the faeces of rats administered [Cl-phenyl-U-^{14}C]flumethrin. When [F-phenyl-U-^{14}C]flumethrin was administered, 3-(4-hydroxy-phenoxy)-4-fluorobenzoic acid and 3-phenoxy-4-fluorobenzoic acid were also identified in urine, accounting for the majority of the radioactivity present. Glycine conjugates of the primary metabolites (each <10% of urinary radiolabel) were also identified (Ecker, 1983). In a dairy cow, flumethrin and flumethrin acid were found in all tissues; in milk, only flumethrin and an unknown metabolite were found. The glucuronidated form of the acid was also detected in the liver and kidney (Gifford & Dunsire, 1994; Klein, 1995).

3.2 Toxicological data

Critical studies relevant to the risk assessment of flumethrin were identified (Table 6). Most of the toxicity studies were conducted under GLP-compliant conditions, and the purity of the test substance used, where specified, was greater than 90%.

The oral acute toxicity of flumethrin in rats was dependent on the vehicle used. When flumethrin was given in peanut oil, the oral LD_{50} was 662 mg/kg bw (Renhof, 1983a); when given in miglyol, the oral LD_{50} was 2248 mg/kg bw (Renhof, 1983a); when given in acetone : peanut oil (1 : 10), the oral LD_{50} was 138 mg/kg bw (Renhof, 1983b); and when given in corn oil (50% v/v), the oral LD_{50} was 175 mg/kg bw (Kuhn, 2007a).

The dermal LD_{50} for flumethrin in corn oil was 1436 mg/kg bw in rats (Kuhn, 2008).

The inhalation LC_{50} for flumethrin was 0.572 mg/L in rats (Pauluhn, 1996).

Flumethrin was not irritating to the skin (Krötlinger, 1994; Kuhn, 2007b) or the eyes of rabbits (Krötlinger, 1994). Flumethrin was not sensitizing to the

Table 6
Studies relevant to risk assessment

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				
Three-month toxicity study (dietary)	Male: 0, 0.9, 1.9, 9.9, 20.8, 42.2, 80 [a] Female: 0, 1.4, 2.5, 13.2, 26.5, 56.0, 100 [a]	Hair loss, piloerection	0.9	1.9
Eighteen-month carcinogenicity (dietary)	Male: 0, 0.12, 0.39, 1.97, 4.56 Female: 0, 0.15, 0.52, 2.34, 4.95	Clinical signs of skin wounds and high stepping gait	Toxicity: 0.39	1.97
		Plasmocytosis, increased lymphoid hyperplasia & enlarged spleens	Carcinogenicity: none	–
Rat				
Thirteen-week toxicity study (dietary)	Male: 0, 0.70, 3.54, 11.11 Female: 0, 0.84, 4.21, 13.24	Inflammatory ulcerative changes in skin	0.7	3.54
Fifteen-week toxicity study (dietary)	Male: 0, 0.7, 2.9, 11.9 Female: 0, 0.8, 3.4, 13.0	Scratching behaviour and skin lesions	0.7	2.9
Two-year study of toxicity and carcinogenicity (dietary)	0, 0.7, 2, 4(6) [b]	Clinical signs of skin changes and hair loss. Increased vacuolation in the adrenal cortex	Toxicity: 0.7 Carcinogenicity: none	2 –
Two-generation reproductive toxicity study (dietary)	Male: 0, 0.07, 0.37, 3.78 Female: 0, 0.08, 0.40, 4.05	Reproductive toxicity: Nil	3.78 [c]	–
		Parental toxicity: Skin lesions	0.37*	3.78
		Offspring toxicity: Reduced pup survival and body-weight gain	0.37*	3.78
Two-generation reproductive toxicity study (gavage)	0, 0.5, 1, 3	Reproductive toxicity: Nil	3 [c]	–
		Parental toxicity: Reduced body weight	1	3
		Offspring toxicity: Reduced pup weight, reduced pup viability and clinical findings	1	3
Developmental toxicity study (gavage)	0, 0.5, 1.0, 2.0	Maternal toxicity: Salivation	0.5**	1.0
		Developmental toxicity: reduced placental weight, reduced fetal weights, increased skeletal variations	1.0	2.0
	0, 0.75, 2, 5	Maternal toxicity: Salivation	0.75	2
		Developmental toxicity: Reduced fetal weights	2	5
Rabbit				
Developmental toxicity study (gavage)	Study 1: 0, 0.5, 1.7, 6.0	Maternal toxicity: Reduced body weight, reduced feed intake	1.7	6.0
		Developmental toxicity: Reduced fetal weights	1.7	6.0
	Study 2: 0, 0.5, 1.5, 4.5	Maternal toxicity: Limb swelling	0.5	1.5
		Developmental toxicity: Increased skeletal variations	1.5	4.5

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)
Dog				
Thirteen-week toxicity study (dietary)	Study 1: Male: 0, 2.1, 4.7, 9.6 Female: 0, 2.3, 5.0, 9.0	Thinned hair/hairless and weeping, ulcerative, scabbed patches on skin	–	2.1 [d]
	Study 2: Male: 0, 0.88 Female: 0, 0.94	Nil	0.88 [c, e]	–

* Pivotal study for the derivation of the ADI (*Dotti et al., 1992*)
** Pivotal study for the derivation of the ARfD (*Kowalski, Clemens & Hartnagel, 1987*)
[a] Estimated dose.
[b] Dose reduced from 6 to 4 mg/kg bw per day from week 18.
[c] Highest dose tested.
[d] Lowest dose tested.
[e] Overall NOAEL.

skin in guinea-pigs in Magnusson and Kligman maximization tests (Diesing, 1991; Vohr, 1994).

The primary target for toxicity was the CNS. Toxicity manifested as reduced motor activity, respiratory disorders, altered gait and salivation in multiple species. The onset of toxicity was short (1–15 minutes after administration), and the effects were comparatively long-lasting. In addition, the CNS-mediated paraesthetic effects common to other alpha-cyano pyrethroids were seen in mice, rats, rabbits and dogs and included intensive grooming and scratching behaviours leading to skin lesions.

In two 3-month dose-ranging studies, mice were fed diets containing flumethrin at concentrations of 0, 5, 10, 60, 120, 240 or 480 mg/kg feed (equal to 0, 0.9, 1.9, 9.9, 20.8, 42.2 and approximately 80 mg/kg bw per day for males and 0, 1.4, 2.5, 13.2, 26.5, 56 and approximately 100 mg/kg bw per day for females, respectively). The mortality was increased at doses of 60 mg/kg feed and greater, with all high-dose animals dying within the first week of treatment. The severity of clinical signs increased with dose, with mild skin changes (e.g. hair loss) at 5 and 10 mg/kg feed and piloerection at 10 mg/kg feed. Key histopathological findings were related to skin ulcers that occurred above 10 mg/kg feed. Mild skin changes were the only pathological findings noted in the 5 mg/kg feed group and were not considered to be adverse.

The overall NOAEL in these two dose range–finding studies in mice was 5 mg/kg feed (equal to 0.9 mg/kg bw per day) based on clinical signs observed at 10 mg/kg feed (Leser & Popp, 1998).

In a 13-week non-GLP-compliant study, rats were fed diets containing flumethrin at concentrations of 0, 10, 50 or 150/250 mg/kg feed (150 mg/kg feed

from the third week onwards) for 13 weeks (equal to 0, 0.70, 3.54 and 11.11 mg/kg bw per day for males and 0, 0.84, 4.21 and 13.24 mg/kg bw per day for females, respectively; highest dose given for 150 mg/kg feed). By 2 weeks, 10% of animals at 50 mg/kg feed and 50% of animals at 250 mg/kg feed showed inflammatory ulcerative skin changes. Unscheduled deaths occurred only in high-dose animals. The NOAEL was 10 mg/kg feed (equal to 0.7 mg/kg bw per day) based on inflammatory ulcerative changes in the skin at 50 mg/kg feed (equal to 3.54 mg/kg bw per day) (Hahnemann & Rühl, 1985).

In a 15-week study, rats were fed diets containing flumethrin at concentrations of 0, 10, 40 or 160 mg/kg feed in the diet (equal to 0, 0.7, 2.9 and 11.9 mg/kg bw per day for males and 0, 0.8, 3.4 and 13.0 mg/kg bw per day for females, respectively). There were no unscheduled deaths. A limited number of animals in the 40 mg/kg feed group (n = 2) had skin lesions, but these were of lesser intensity compared with high-dose animals and were generally reversible. The NOAEL was 10 mg/kg feed (equal to 0.7 mg/kg bw per day) based on isolated and reversible cases of scratching behaviour and skin lesions at 40 mg/kg feed (equal to 2.9 mg/kg bw per day) (Bomann & Sander, 1995).

In two non-GLP-compliant studies, dogs were fed diets containing flumethrin at concentrations of 0, 25, 50, 100 or 200 mg/kg feed (equal to 0, 0.88, 2.1, 4.7 and 9.6 mg/kg bw per day for males and 0, 0.94, 2.3, 5.0 and 9.0 mg/kg bw per day for females, respectively) for 13 weeks. There were no treatment-related deaths. Animals receiving 50 mg/kg feed and higher concentrations showed partial or complete hair loss and, in some instances, weeping ulcerative scabbed patches on the neck, back, tail, ears and limbs that had partially healed by the end of the study.

The overall NOAEL for the two 13-week dog studies was 25 mg/kg feed (equal to 0.88 mg/kg bw per day) based on skin changes at 50 mg/kg feed (equal to 2.1 mg/kg bw per day) (Hoffmann & Kaliner, 1984; Hoffmann, 1985).

In an 18-month study, mice were fed diets containing flumethrin at concentrations of 0, 1, 3, 15 or 30 mg/kg feed (equal to 0, 0.12, 0.39, 1.97 and 4.56 mg/kg bw per day for males and 0, 0.15, 0.52, 2.34 and 4.95 mg/kg bw per day for females, respectively). Clinical signs of toxicity in the 1 and 3 mg/kg feed groups were limited to slight and transient red spots on the tails of both sexes. At 15 mg/kg feed, animals were observed with auricle loss, wounds on hairless areas and high stepping gait. Non-neoplastic changes (enlarged mandibular lymph nodes, plasmacytosis, increased lymphoid hyperplasia, enlarged or swollen spleens, slight increase in the incidence of diffuse glandular hyperplasia in stomach, increased incidence of chronic nephropathy, decreased hepatocellular glycogen content and increased hepatocellular atrophy) were considered secondary to the skin changes and poor condition of the higher-dose treatment groups. There were

no dose-related or sex-related changes in the incidence of benign or malignant tumours or the total number of tumours.

The NOAEL was 3 mg/kg feed (equal to 0.39 mg/kg bw per day) based on clinical signs and non-neoplastic histopathological findings at 15 mg/kg feed (equal to 1.97 mg/kg bw per day) (Wirnitzer & Hartmann, 1999).

In a 2-year combined chronic toxicity and carcinogenicity study, rats were fed diets containing flumethrin at concentrations of 0, 0.7, 2 or 4 (reduced from 6 at week 18) mg/kg bw per day. Severe skin changes (wounds) resulted in a number of animals at 6 mg/kg bw per day being euthanized in week 17 of treatment. The highest dose was subsequently reduced to 4 mg/kg bw per day from week 18 in all surviving high-dose animals. Incidental skin changes were limited to only two animals at 0.7 mg/kg bw per day. With increasing dose, clinical signs of toxicity included hair loss and skin changes at 2 mg/kg bw per day and piloerection and more extensive hair loss and skin changes at 4 mg/kg bw per day. Increased vacuolation in the adrenal cortex was noted in males at doses of 2 and 4 mg/kg bw per day. There were no significant organ weight changes. Histopathological changes noted at necropsy were related to the poor condition of the animals. There were no treatment-related increases in benign or malignant tumours in either sex. The NOAEL was 0.7 mg/kg bw per day, based primarily on clinical findings at 2 mg/kg bw per day (Schladt & Deschl, 1999).

The Committee concluded that flumethrin is not carcinogenic in mice or rats.

The genotoxicity of flumethrin was evaluated in an adequate range of assays, both in vitro and in vivo. No evidence of genotoxicity was found, other than in one mouse micronucleus study (Nakano, Rabello-Gay & Pereira, 1996). The potential for genotoxicity reported in this study was inconsistent with other studies, and is likely to be related to the low purity of flumethrin in the study (~60% versus >90% in most other studies).

The Committee concluded that flumethrin is unlikely to be genotoxic.

In view of the lack of genotoxicity and the absence of carcinogenicity in mice and rats, the Committee concluded that flumethrin is unlikely to pose a carcinogenic risk to humans.

In a two-generation reproductive toxicity study, rats received diets containing flumethrin at concentrations of 0, 1, 5 or 50 mg/kg feed (equal to 0, 0.07, 0.37 and 3.78 mg/kg bw per day in F_0 males and 0, 0.08, 0.40 and 4.05 mg/kg bw per day in F_0 females, respectively). No treatment-related clinical signs were noted at either 1 or 5 mg/kg feed in any generation. After treatment with 50 mg/kg feed, skin lesions developed in F_0 male and female animals and in F_1 females. Body weights of F_0 males and F_1 males and females were reduced in all phases. Survival in all F_1 and F_2 animals at 50 mg/kg feed was lower during the first 4 days after birth, and body-weight gain was reduced. A higher incidence

of pups with hypothermia and with cramped or hunched posture, stiff limbs in caudal posture and/or pectus carinatum (pigeon chest) was also seen at 50 mg/kg feed; vocalization was also more frequent. These observations were probably secondary to the toxic effects on the parents. No malformations were found. The NOAEL for parental toxicity was 5 mg/kg feed (equal to 0.37 mg/kg bw per day) based on skin lesions at 50 mg/kg feed. The NOAEL for offspring toxicity was 5 mg/kg feed (equal to 0.37 mg/kg bw per day) based on reduced survival and body-weight gain in pups at 50 mg/kg feed. The NOAEL for reproductive toxicity was 50 mg/kg feed (equal to 3.78 mg/kg bw per day), the highest dose tested (Dotti et al., 1992).

In a two-generation reproductive toxicity study, rats were administered flumethrin, via gavage, in corn oil vehicle at doses of 0, 0.5, 1 or 3 mg/kg bw. Unscheduled deaths were unrelated to the test substance, and clinical signs were generally unremarkable and limited to a small number of high-dose animals with hair loss and alopecia in F_0 animals. At 3 mg/kg bw, F_0 and F_1 males and F_1 females had significantly lower body weights compared with controls, and F_0 and F_1 dams had a reduced feed intake. There were no other significant treatment-related effects in these animals. Significant clinical findings in pups were limited to the high dose group, in both F_1 and F_2 pups, and included a reduction in pups and litters with no milk spots and thin, relatively small appearance. These findings were concordant with significantly reduced mean pup weight throughout the lactation period in F_1 and F_2 pups. Sexual maturation in F_1 pups was slightly delayed. The NOAEL for parental toxicity was 1 mg/kg bw per day based on reduced body weight at 3 mg/kg bw per day. The NOAEL for offspring toxicity was 1 mg/kg bw per day based on reduced pup weight, reduced pup viability and clinical findings at 3 mg/kg bw per day. The NOAEL for reproductive toxicity was 3 mg/kg bw per day, the highest dose tested (Eiben, 2008).

In a developmental toxicity study, rats were administered flumethrin, via gavage, in distilled water containing 5% Emulphor EL-719 (ethoxylated castor oil) and 5% ethanol vehicle at 0, 0.5, 1.0 or 2.0 mg/kg bw per day from gestation day 6 to 15. There were no unscheduled deaths. At 1.0 mg/kg bw per day, some dams exhibited salivation from the first day of dosing. At 2.0 mg/kg bw per day, hypoactivity, ptosis, ataxia, lachyrmation and urine-stained ventral surface were also evident. In addition, the high-dose dams had a reduced mean feed consumption, mean body weight and mean body-weight gain. Fetotoxicity at 2.0 mg/kg bw per day was noted as a slight but significant reduction in median placental weights (0.48 g versus 0.53 g in controls) and median combined fetal weights (3.4 g versus 3.8 g in controls) and an increased incidence of reduced ossification at several sites, notably the skull bones (67% versus 42% in controls) and cervical vertebral arches (16% versus 1% in controls). The NOAEL for maternal toxicity was 0.5 mg/kg bw per day based on clinical signs at 1.0 mg/

kg bw per day. The NOAEL for embryo/fetal toxicity was 1.0 mg/kg bw per day based on reduced placental weight, reduced fetal weights and an increase in the incidence of skeletal variations at 2.0 mg/kg bw per day. There was no evidence of teratogenicity (Kowalski, Clemens & Hartnagel, 1987).

In a developmental toxicity study, rats were administered flumethrin, via gavage, in corn oil at 0, 0.75, 2 or 5 mg/kg bw per day from gestation day 6 to 19. Salivation was evident in dams at 2 and 5 mg/kg bw per day. Body-weight loss was noted in the high-dose dams at gestation day 6–7, with reduced body-weight gain during the rest of the treatment period. Placental weight was significantly lower in high-dose dams (0.47 g versus 0.61 g in controls). Fetal weights from high-dose dams were also significantly reduced compared with controls (2.79 g versus 3.62 g), and were below the historical control range. There were no significant external or visceral changes in fetuses. Delays in skeletal ossification and increased variations, noted mainly at the high dose, were not considered toxicologically significant as they were concordant with delayed development from reduced fetal weights. The NOAEL for maternal toxicity was 0.75 mg/kg bw per day based on salivation at 2 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 2 mg/kg/bw per day based on reduced fetal weights at 5 mg/kg bw per day. There was no evidence of teratogenicity (Klaus, 1999).

In a developmental toxicity study, rabbits were administered, via gavage, flumethrin in ethoxylated castor oil/ethanol vehicle at 0, 0.5, 1.7 or 6.0 mg/kg bw per day from gestation day 7 to 19. There were no unscheduled deaths, although two low-dose and two high-dose does aborted between gestation day 18 and 23. There were no significant clinical signs in any of the treated does. Animals at 6 mg/kg bw per day had reduced feed intake and reduced body weights during the treatment period. At the high dose, there was a slight trend for a reduction in fetal weights (33.9 g versus 35.9 g in controls) that was lower than the testing laboratory historical control range (34.7–39.8 g). There were no statistically or toxicologically significant changes in the incidence of skeletal, external and visceral changes or malformations/variations in the fetuses. The NOAEL for maternal toxicity was 1.7 mg/kg bw per day based on reduced feed intake and body weight at 6.0 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 1.7 mg/kg bw per day based on reduced fetal weights at 6.0 mg/kg bw per day. There was no evidence of teratogenicity (Clemens & Hartnagel, 1987).

In another developmental study, rabbits were administered, via gavage, flumethrin in corn oil at 0, 0.5, 1.5 or 4.5 mg/kg bw per day from gestation day 6 to 28. There were no unscheduled deaths in the does. Four high-dose does aborted during gestation days 25 to 29. Limb swelling was apparent at both 1.5 and 4.5 mg/kg per day. The incidence of fused sternebrae was statistically significantly increased in the high-dose group on a fetal basis (17.9% versus 3.8% in controls), but not on a litter basis (47.1% versus 25% in controls) although this was at the

upper end of the historical control range (47.1%) for this strain of rabbit. The NOAEL for maternal toxicity was 0.5 mg/kg bw per day based on clinical signs at 1.5 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 1.5 mg/kg bw per day based on an increase in skeletal variations at 4.5 mg/kg bw per day. There was no evidence of teratogenicity (Langewische, 2009).

The Committee concluded that flumethrin is not teratogenic in rats or rabbits.

3.3 Observations in humans

There was limited information available on flumethrin-induced toxicity in humans.

3.4 Microbiological data

Considering the chemical structure and mode of action of flumethrin, the Committee did not anticipate any adverse effects of flumethrin residues on human gastrointestinal microbiota.

4. Evaluation

The Committee established an ADI of 0–0.004 mg/kg bw on the basis of a NOAEL of 0.37 mg/kg bw per day for skin lesions in parental animals and reduced survival and body-weight gain in pups in a two-generation toxicity study in rats, with application of a safety factor of 100 to account for interspecies and intraspecies variability, and rounded to one significant figure.

The Committee established an acute reference dose (ARfD) of 0.005 mg/kg bw on the basis of a NOAEL of 0.5 mg/kg bw for salivation in dams in a developmental toxicity study in rats, with application of a safety factor of 100.

In view of the toxicological profile of flumethrin, specific exposure scenarios are required to address exposure to pregnant women, infants and children, and high percentile adult consumers.

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Halquinol

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1. Explanation

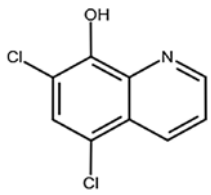
Halquinol (Chemical Abstract Service number 8067-69-4) is composed of a mixture of chlorinated products of quinolin-8-ol (Fig. 1). Chlorinating quinolin-8-ol yields a mixture that contains 5,7-dichloroquinolin-8-ol (5,7-DCL or DCHQ; 57–74% weight per weight [w/w]), 5-chloroquinolin-8-ol (5-CL or

CHQ; 23–40% w/w) and 7-chloroquinolin-8-ol (7-CL; 0–4% w/w).[1] Halquinol is a quinoline with broad spectrum antimicrobial activity but a different mode of action than quinolones.

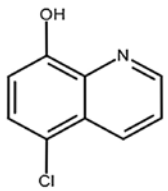
The halquinol formulation approved for veterinary use in medicated feed consists of 60% halquinol with silicon dioxide and chalk as inert excipients. It is indicated for use in poultry and swine for the enhancement of feed efficiency, and in swine for the control, treatment and prevention of scours/diarrhoea caused or complicated by *Escherichia coli* and *Salmonella* spp. The approved halquinol inclusion rates in swine feed range from 60 to 600 mg/kg feed for up to 15 consecutive days. Based on an estimated daily feed consumption in swine of approximately 4% body weight, the resulting daily halquinol dose is approximately 2.4–24 mg/kg body weight (bw). Withdrawal periods for approved halquinol products range from 0 to 7 days.

Halquinol has not previously been evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). The Committee evaluated halquinol at the request of the Codex Committee on Residues of Veterinary Drugs in Foods at its Twenty-third Session, with a view to establishing relevant health-based guidance values and recommending maximum residue limits in swine tissues (FAO/WHO, 2016).

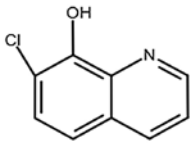
Fig. 1
Structural formula of main components of halquinol



5,7-dichloroquinolin-8-ol (5,7-DCL)



5-chloroquinolin-8-ol (5-CL)



7-chloroquinolin-8-ol (7-CL)

The present Committee considered data on pharmacokinetics, short-term toxicity and long-term toxicity, genotoxicity, reproductive and developmental toxicity and microbiological safety. The studies submitted by the sponsor were all performed in 2013–2017, and therefore most comply with good laboratory practice (GLP) standards and/or Organisation for Economic Co-operation and

[1] These names, as well as 5,7-dichloro-8-hydroxyquinoline and 5,7-dichloro-8-quinolinol, for 5,7-dichloroquinolin-8-ol, and 5-chloro-8-hydroxyquinoline for 5-chloroquinolin-8-ol, are used interchangeably in this monograph.

Development Test Guidelines (OECD TG) and USFDA GLP for Nonclinical Laboratory Studies.

A literature search of the PubMed and Embase databases was conducted using the keywords "halquinol", "quixalud", "chloroxine", "quixalin", "cloroquinol", "5-chloroquinolin-8-ol", "7-chloroquinolin-8-ol", "5,7-dichloroquinolin-8-ol", "5,7-dichloro-8-hydroxyquinoline" and "5-chloro-8-hydroxyquinoline". The 106 retrieved references were manually screened. Of these, 19 reports were found to be relevant and are cited in the current monograph.

2. Biological Data

2.1 Biochemical aspects

2.1.1 Absorption, distribution and excretion

Halquinol is a mixture of three chloro-8-hydroxyquinoline-based compounds. No studies provide absorption, distribution and excretion information for all the three compounds. Because of their structural similarities, the disposition of the compounds is assumed to be similar. Read-across from one to another was considered possible as the disposition of other structurally related halogenated 8-hydroxyquinolines, such as 5-chloro-7-iodo-quinolin-8-ol (ICHQ), 5-CL and 5,7-dibromoquinolin-8-ol are qualitatively similar if quantitatively different.

Haskins & Luttermoser (1953) showed that rabbits excreted a total of 30–50% of a 20 mg/kg bw per day dose of ICHQ in urine following oral exposure for 6 days.

Differences in absorption, metabolism and excretion of ICHQ in male Donryu rats, male Hartley guinea-pigs and humans (sex not specified) have been investigated. This investigation and a follow-up study with 8-hydroxyquinolone (Kiwada et al., 1977) demonstrated that all orally administered hydroxyquinolines underwent extensive first-pass metabolism. However, the extent and type of conjugation varied between species. Whereas rats excreted 13% of predominantly sulfated ICHQ conjugates (10%) in urine following a 50 mg/kg bw gavage dose of ICHQ, Hayashi et al. (1976) demonstrated that guinea-pigs excreted around 42% of a similar oral dose, predominantly in the form of ICHQ glucuronide (34%). In humans, the glucuronide (16%), but not the ICHQ sulfate conjugate, was detected in urine following an oral dose of approximately 10 mg/kg bw.

Only ICHQ glucuronide (30%) was detected in rat bile; in guinea-pigs, only 6% of the dose was found as a sulfated ICHQ conjugate (Hayashi et al., 1976). Kotaki et al. (1983) observed a similar excretion profile in rats following

intraduodenal administration of ICHQ at 10 mg/kg bw: the amount of sulfated ICHQ conjugate and ICHQ glucuronide excreted in urine and bile after 24 hours was 10% and 38%, respectively.

In another study at the same laboratory, Kiwada et al. (1977) investigated the kinetics of 8-hydroxyquinoline, the parent compound of ICHQ, in male Donryu rats after intravenous administration of [^{14}C]8-hydroxyquinoline (~17 mg/kg bw). Bile (via biliary fistula) and urine samples were collected at intervals for 8 hours while blood was collected for 4 hours. Urinary metabolites were predominately 8-hydroxyquinoline glucuronide conjugates (65%) and 8-hydroxyquinoline sulfates (23%). This contrasts with the ratio of ICHQ conjugate excreted in urine following intravenous dosing, that is, 35% sulfate conjugates versus 6% glucuronides. In bile, the sulfate conjugate was not detected for either 8-hydroxyquinoline or ICHQ; all the conjugates excreted in bile were in the form of glucuronides (8-hydroxyquinoline: 9%; ICHQ; 32%). There was evidence for enterohepatic recirculation after cannulation of the bile duct; the "shoulders" were no longer apparent in the concentration–time curve for the sulfate and glucuronide conjugates. The study authors stated that it was not possible to determine a plasma half-life for either conjugated or unconjugated 8-hydroxyquinoline following intravenous dosing due to the non-linearity of the semi-log concentration–time curve.

Sawada et al. (1978) observed that while most of an 11 mg/kg bw intravenous dose of glucuronidated 5,7-DCL was excreted in the bile (61%) and urine (13%) after 8 hours, around 21% was metabolized to the sulfated conjugate and excreted in urine. Similarly, for intravenously administered sulfated 5,7-DCL, most (86%) was excreted in urine but around 4% of glucuronidated 5,7-DCL was excreted in urine and a further 8% in bile. Similar metabolic conversion of intravenously administered sulfated 5-CL (~11 mg/kg bw) was also observed: approximately 65% of sulfated 5-CL was excreted in urine but a further 16% and 6% of glucuronidated 5,7-DCL was excreted in urine and bile, respectively. For glucuronidated 5-CL conjugate, 58% was excreted in urine and 31% in bile, but no sulfated 5-CL was found in urine or bile. These data suggest that both sulfated and glucuronidated conjugates can be hydrolysed in vivo.

Bories & Tullierz (1972) investigated the disposition of ^{36}Cl-radiolabelled DCHQ and CHQ (ratio 79 : 21) in male Wistar rats and a Friesian calf. Following gavage doses of 15 mg/kg bw in rats, more than 90% was excreted in either urine (26%) or faeces (69%) within 48 hours after dosing; 99.7% of the administered dose could be recovered in excreta over 8 days. In contrast, 80.5% of the radioactivity was excreted in calf urine after an oral dose of 3 mg/kg bw by stomach intubation and only about 10.5% was in faeces. The overall excretion rate was slower with radioactivity appearing in urine over 5–6 days. In a separate study, male rats treated with radiolabelled DCHQ and CHQ at 80 mg eq./kg in the feed for up to

15 days showed no evidence of bioaccumulation in liver, kidneys, muscle or fat. Similarly, rapid depletion of radioactivity from these tissues was observed over 36 hours in male rats after administering unlabelled DCHQ and CHQ (100 mg/kg) for 15 days followed by another 8 days in which the radioactivity (100 mg eq./kg) was added to the feed. Tissue residues in the calf were low with the highest levels being found in kidney and liver.

Investigated the pharmacokinetics of ICHQ in humans, Jack & Reiss (1973) reported a plasma half-life of between 10 and 14 hours following oral doses of between approximately 4 and 25 mg/kg bw. In six volunteers, up to 25% of CHQ at an oral dose of 750 mg was excreted in urine over 72 hours in the form of the glucuronide (Berggren & Hansson, 1968).

2.1.2 Biotransformation

In a GLP-compliant study, Novo (2015a,b) examined the comparative metabolism of [^{14}C]5,7-DCL in cryopreserved hepatocytes and hepatic microsomes prepared from Sprague Dawley rats, beagle dogs, Göttingen mini-pigs, Landrace pigs and humans.

[^{14}C]5,7-DCL was incubated with cryopreserved hepatocytes or hepatic microsomes (single sex) prepared from each of the species. The chemical authenticity of [^{14}C]5,7-DCL was confirmed by chromatography, and its specific activity was 3489.1 MBq (94.3 mCi/mmol). These were 2 times the final incubation concentration; [^{14}C]5,7-DCL concentrations in the incubations were 5 and 20 µmol/L. [^{14}C]5,7-DCL solutions used in the incubations with hepatic microsomes were prepared at 0.5 and 1 mmol/L concentrations in ethanol. These concentrations were 100 and 50 times higher than the final incubation concentration, respectively, such that [^{14}C]5,7-DCL was dosed at 5 and 20 µmol/L and the solvent concentrations were 1% and 2%, respectively.

Incubations with hepatocytes were performed in duplicate in 12 well plates in an incubator set to maintain a temperature of 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Incubations of [^{14}C]5,7-DCL (5 or 20 µmol/L) and hepatocytes (1×10^6 cells/mL) in a final volume of 1 mL of supplemented DMEM (Dulbecco's Modified Eagle Medium) were terminated after 0, 60 or 240 minutes by transferring an aliquot (100 µL) from each well into tubes containing 100 µL of ice cold acetonitrile. The remaining volume left in each well (~700 µL) was terminated in tubes containing 700 µL of ice cold acetonitrile immediately after removal of the last 100 µL aliquot at 240 minutes. These samples provided material for high-performance liquid chromatography (HPLC) determination. Blank incubations were performed in parallel, in the absence of hepatocytes to test the stability of the test item under the incubation conditions. The metabolic competence of the hepatocyte preparations (1×10^6 cells/mL) was determined in

incubations with [^{14}C]7-ethoxycoumarin (10 µmol/L) and [^{14}C]testosterone (150 µmol/L) in a final volume of 1 mL of supplemented DMEM.

Incubations with hepatic microsomes were performed in duplicate at 37 °C. Incubations contained microsomal protein (1 mg/mL), [^{14}C]5,7-DCL (5 or 20 µmol/L) and potassium phosphate buffer (50 mmol/L, pH 7.4) in a final volume of 1000 µL. Following 2 minutes pre-warming, reactions were initiated by addition of nicotinamide adenine dinucleotide phosphate (reduced; NADPH; 1 mmol/L). Incubations were terminated after 0, 60 and 120 minutes by transferring an aliquot (200 µL) of each incubation mix into tubes containing 200 µL of ice cold acetonitrile. At 240 minutes, the remaining samples (400 µL) were terminated by adding them to tubes containing equal volume of ice cold acetonitrile. These samples provided material for analytical method development. Blank incubations containing heat-inactivated microsomes from a single species (human) were performed in parallel. The metabolic competence of the microsomal preparations was assessed in incubations performed under the same conditions with the probe substrate of [^{14}C]testosterone (100 µmol/L).

Analysis of the postincubation supernatants containing [^{14}C]5,7-DCL parent and metabolites was performed by HPLC, with 100% of the eluent flow from the HPLC diverted to the radiodetector. For liquid chromatography–mass spectrometry (LC-MS), 75% of the eluent flow from the HPLC was diverted to the radiodetector, 25% to the mass spectrometer. The radioactive content of the dosing solutions and samples from incubations performed with 5 µmol/L [^{14}C]5,7-DCL was assessed by liquid scintillation counting (LSC).

The rate of test item metabolism was determined by HPLC with online radiodetection. Each replicate sample from incubations of rat, dog, mini-pig, pig or human hepatocytes and microsomes with [^{14}C]5,7-DCL (20 µmol/L) was determined by LC-MS. The viability of male and female hepatocytes from these species and strains was determined using the Trypan blue exclusion method. The viability of female human hepatocytes was 67%; the viabilities of all the other hepatocytes were over 70%. Metabolic competence assays confirmed that the hepatocytes were suitable for use.

[^{14}C]5,7-DCL was extensively metabolized to several metabolites in the samples tested. Complete metabolism (i.e. no parent compound remaining at the end of the incubation period of 240 minutes) was observed in hepatocytes from male and female mini-pig, pig and rat where only 7.5% of test item remained at the end of the incubation period. In dog and male human hepatocytes, metabolism was statistically significantly lower, with about 50% and 55%, respectively, remaining at 240 minutes. In female mini-pig and pig hepatocytes, [^{14}C]5,7-DCL was extensively metabolized (~5.5% and 15%, respectively, of parent compound remaining at 60 minutes). Metabolism was also extensive in female rat and dog hepatocytes (13.4% and 11.3%, respectively, of parent compound remaining at

240 minutes, respectively). The metabolism of the test item was lowest in human hepatocytes (36.1% of parent compound remaining at 240 minutes); however, the rate of metabolism in female cells was higher than that observed in male human hepatocytes.

Two major polar metabolites, designated M1 and M2, eluting at about 19.5 and 20.4 minutes, respectively, were detected in all samples except male and female human hepatocytes, where only one metabolite, eluting at about 20.4 minutes, was observed.

A major metabolite common to all species and sexes that eluted at about 22.6 minutes was designated M3. In male rat and female human microsomes, only M3 was present. In all other species and sexes, metabolic profiles were more complex, with several metabolites detected.

Microsome incubations resulting in no detection of [^{14}C]5,7-DCL at the end of the incubation period were observed in dog, male and female pig and female mini-pig. The lowest rate of test item metabolism was observed in male and female rat microsomes – 19.6% and 26.8%, respectively. The rates of metabolism observed in rat hepatocytes were significantly higher (92.6% in males and 86.7% in females) suggesting a preference for phase II metabolism of 5,7-DCL in this species.

LC-MS determination of high-dose samples (20 µmol/L) revealed four 5,7-DCL metabolites: oxidized parent compound (M3); glucose conjugate (M1); glucuronide conjugate (M2); and two isomers of dichloro hydroxyl conjugates of 5,7-DCL. One peak at about 14 minutes was also present in some samples, but this could not be identified due to lack of clear spectra.

The two major metabolites common to all the tested hepatocyte samples were a glucose conjugate and a glucuronide conjugate. In hepatic microsomes, hydroxy-5,7-dichloro-8-quinolinol was the major metabolite common to all samples except the rat. In rat microsomes, HPLC determination revealed a peak at about 22.3 minutes, which is consistent with the hydroxy-5,7-dichloro-8-quinolinol peak observed in another species. However, this peak was not present in the high-dose samples used for metabolite identification.

Oxidative de-chlorination and glucuronide conjugation were also observed in mini-pig and pig hepatocytes. The metabolites detected in the human samples were also present in the other species.

2.2 Toxicological studies

2.2.1 Acute toxicity

(a) Lethal doses

In studies that predate the implementation of GLP, mice and rats were administered halquinol in a single oral dose. No information about the number

or strain of animals or other experimental conditions was provided. The oral median lethal dose (LD_{50}) was 470–850 mg/kg bw in mice and 700 mg/kg bw in rats. In mice, reported LD_{50} values were between 100 mg/kg bw and 1300 mg/kg bw, depending on the strain (Boissier, 1959; Eckert, 1961; Newman, 1963; Hurd, 1964; Trabucchi, 1964; Adams, 1973; Tateishi et al., 1975).

(b) Acute toxicity

In a preliminary dose range–finding toxicity study to select the highest dose for a bone marrow micronucleus induction study, Sire (2014a) administered halquinol BP 80 (26.29% of 5-CL : 72.53% of 5,7-DCL) in 0.5% methylcellulose in deionized water vehicle orally to male Sprague Dawley rats (5/group) at a single dose of 0, 500, 1000 or 2000 mg/kg bw for sampling time at 18 hours.

There were no deaths. No clinical signs of toxicity were observed at 500 or 1000 mg/kg bw. At 2000 mg/kg bw, one out of three animals had piloerection 2 hours after the single treatment.

2.2.2 Short-term studies of toxicity

(a) Rats

In a 28-day oral toxicity study without a GLP-compliance statement, Swetha (2007) administered halquinol BP 80 (26% of 5-CL : 72% of 5,7-DCL) in tragacanth mucilage vehicle by gavage to adult female Wistar rats (6/group) at 0, 150, 450 or 1000 mg/kg bw per day. The animals were observed at least twice a day for signs of toxicity. Blood samples were collected for haematological and biochemical analyses. After 28 days, the animals were killed, organs weighed and gross and histopathological examination of tissues performed.

Rats at 450 and 1000 mg/kg bw per day showed burrowing behaviour following dosing. Over the duration of the study, all test groups showed a reduction in body-weight gain (by 6%, 52% and 78%, respectively; $P < 0.01$ at 450 and 100 mg/kg bw per day). At 1000 mg/kg bw per day, haemoglobin concentration, total red blood cell (RBC) count and mean corpuscular haemoglobin concentration decreased significantly, whereas mean corpuscular volume increased significantly. The increase in relative liver and kidney weights reported at 400 and 1000 mg/kg bw per day could be attributed to a significant body-weight loss in these two groups. Although there were no treatment-related macroscopic changes in tissues at 400 and 1000 mg/kg bw per day, the changes in liver histopathology (focal necrosis, congestion, hydropic degeneration) and kidney (cystic dilation of collecting tubules, necrosis of tubular epithelial lining) were associated with significant increases in several clinical chemistry parameters (alanine transaminase [ALT], alkaline phosphatase [ALP], blood urea nitrogen [BUN] and creatinine). At 400 mg/kg bw per day, the histopathological lesions in

liver and kidney were similar to but less severe than those observed at 1000 mg/kg bw per day.

The no-observed-adverse-effect level (NOAEL) was 150 mg/kg bw per day based on changes in liver histopathology and kidney associated with a significant increase in clinical parameters at 450 mg/kg bw per day (Swetha, 2007).

In a GLP-compliant 4-week oral toxicity study conducted in accordance with OECD TG 407, Chevalier (2015) administered halquinol BP 80 (26% of 5-CL : 72% of 5,7-DCL) in 0.5% weight per volume (w/v) methylcellulose to Sprague Dawley rats (5/sex per group) by gavage at doses of 0, 150, 450 or 750 mg/kg bw per day. A constant dose volume of 5 mL/kg bw was used in all groups. In each dose group, blood from an additional three male and three female rats was sampled for toxicokinetic analysis. For the control group, blood was collected 1 hour post dosing on day 1 and 28. For the other groups, blood was collected before dosing and then 1, 3 and 24 hours post dosing on days 1 and 28. The plasma concentration of halquinol and its metabolites was determined using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS).

Animals were observed daily for clinical signs. Feed consumption and body weights were measured weekly. A functional observational battery involving detailed measurements in reactivity to manipulations and different stimuli, and motor activity, were performed at week 4 (day 23). Haematological parameters (haematocrit, clotting time, total leukocyte count, differential leukocyte count, haemoglobin, RBC count, platelets) and clinical chemistry (electrolytes and enzymes) were determined in blood samples collected at termination. Urine analysis of individually collected urine was performed at the end of treatment (week 4). At necropsy of main study animals, organs weights and macroscopic (all groups) and microscopic examinations (control and high-dose groups) were performed. In addition, kidneys and forestomach of low- and mid-dose females were examined macro- and microscopically.

Toxicokinetic investigations at 750 mg/kg bw per day showed that maximum plasma concentration (C_{max}) was reached at 1 hour post dosing (except for two metabolites, 5,7-dichloro-8-hydroxyquinoline glucuronide and 5,7-dichloro-8-hydroxyquinoline sulfate, with C_{max} at 3 and 6 hours). All plasma samples analysed from control animals were below the lower limit of quantification (LLOQ; <9 ng/mL for 5-CL, <45 ng/mL for 5,7-DCL and <225 ng/mL for metabolites). There were generally no sex differences, except for the two metabolites 5-CL and 5,7-dichloro-8-hydroxyquinoline sulfate, for which plasma exposures were higher in males than in females. Area under the concentration–time curve (AUC) in females was higher than in males for 5,7-DCL and its metabolite 5,7-dichloro-8-hydroxyquinoline glucuronide. No accumulation was observed except for the 5-chloro-8-hydroxyquinoline sulfate and 5,7-dichloro-

8-hydroxyquinoline sulfate (males and females) and 5,7-DCL (females). An apparent increase in clearance was observed for 5,7-DCL and 5,7-dichloro-8-hydroxyquinoline glucuronide in males. The AUC of 5,7-DCL in males was 10- to 25-fold lower than the AUC of the metabolites. In females, the AUC of 5,7-DCL was 5-fold lower than the AUC of the metabolites. Like the dichloro-compound, 5-CL, was rapidly metabolized to 5-chloro-8-hydroxyquinoline glucuronide and 5-chloro-8-hydroxyquinoline sulfate; after 1 hour the parent drug represented less than 1% of the metabolites.

Both groups treated with halquinol at 450 mg/kg bw per day and higher had green faeces. No effects on feed consumption in any groups treated with halquinol BP 80 were seen. There were no treatment-related effects on body-weight gain, except for sporadically reduced body-weight gain. Changes in haematological parameters included decreases in total RBC count, haemoglobin and reticulocyte count at all doses in males and decreases in white blood cells (WBCs) and lymphocytes in females. In males, there were decreases in urea, cholesterol, triglycerides and protein concentrations and ALT activity at all doses and total bilirubin at 450 and 750 mg/kg bw per day. In females, decreases in total bilirubin and triglycerides were observed at all doses.

Appearance of urine changed from slightly turbid to turbid in a dose-related manner at all doses in males and from 450 mg/kg bw per day in females. Generally, clear urine was observed in a few rats at the two highest doses; in these groups, the colour of urine generally differed from that of the control. Although the occasional presence of blood and ammonium phosphate crystals in urine of treated animals was not always dose related, this effect cannot be discounted as unrelated to halquinol treatment as these differences correlated with histopathological findings in kidneys. Compared with control, mean absolute and relative kidney weights were statistically significantly increased in females at 750 mg/kg bw per day (+32%); at 150 and 450 mg/kg bw per day, increases were nonsignificant (+16% and +12%, respectively). Males had nonsignificant increases in mean absolute and relative kidney weights at 150 (+8%), 450 (+8%) and 750 (+8%) mg/kg bw per day; the only statistically significant increase was the relative kidney weight at 450 mg/kg bw per day. Mean absolute and relative thymus weight were decreased in females at 150 (−1%), 450 (−18%) and 750 (−20%) mg/kg bw per day and in males at 750 (−7%) mg/kg bw per day. In males, increased mean absolute and relative adrenal weights (up to +27%) at all dose levels and decreased mean heart weights at 450 and 750 mg/kg bw per day were also observed.

Histopathological examination of kidneys showed tubular and papillary microscopic findings in all females at 450 and 750 mg/kg bw per day. These were characterized by increased severity of tubular basophilia (in the cortex and medulla), minimal to moderate tubular dilation (in the cortex, medulla and

papilla) and slight interstitial oedema of the renal papilla. The severity of the changes was dose related. There were no significant renal microscopic findings in females at 150 mg/kg bw per day. In the forestomach of females, a slight multifocal hyperplasia of the squamous cell mucosa along with minimal diffuse hyperkeratosis was observed in 3/5 females at 750 mg/kg bw per day.

Dark discoloration of the portal lymph nodes was seen at 450 and 750 mg/kg bw per day in males and at all dose levels in females. Minimal infiltrates of macrophages were noted in most of the portal lymph nodes sampled, at all dose levels; however, no control lymph node was obtained for comparison. A trend towards decreased development of germinal centres (decrease size and numbers) in mesenteric lymph nodes and moderate lymphoid atrophy was noted in females at 750 mg/kg bw per day.

No NOAEL could be identified as effects were observed at all doses. 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 changes in kidney histopathology and in lymph nodes at the lowest dose level tested (Chevalier, 2015).

In a GLP-compliant 13-week oral toxicity study with a 4-week recovery period conducted in accordance with OECD TG 408, Bentz (2015a) treated Sprague Dawley (Crl CD) rats (10/sex per group; with an additional 6/sex in the control and 750 mg/kg bw dose groups for a 4-week recovery period) with halquinol BP 80 (purity 98.82%; 26% of 5-CL : 72% 5,7-DCL) by gavage at 0, 50, 150 or 450 mg/kg bw per day. The test item concentrations in the dose formulations were analysed and found to be within acceptable ranges (±15%). No test item was detected in control formulations. The doses were selected based on the results of the Chevalier (2015) 4-week toxicity study. A constant dose volume of 5 mL/kg bw was used. In addition, venous blood samples for toxicokinetic investigations were collected from all satellite animals on day 1 (1 hour post dosing in controls and before dosing and at 1, 3, 6 and 24 hours post dosing in treated groups) and week 13 (1 hour post dosing in controls and before dosing and 1 hour post dosing in treated groups). Satellite animals were killed after the last sampling and discarded without necropsy except for those animals found dead.

Animals were observed daily for physical and behavioural signs of toxicity. Feed consumption and body weights were measured weekly. Functional observational battery (detailed clinical observation, reactivity to manipulations and different stimuli, and motor activity) were performed prior to treatment and at week 12. Opthalmological examinations (of eyelids, conjunctiva and sclera; of cornea, iris, lens and fundus with an ophthalmoscope) were performed at treatment onset in all animals and at week 12 in control and high-dose animals. Haematological parameters and clinical chemistry were determined at 12 weeks

and after the recovery period. Urine collected individually from all main study male and female rats in each group at the end of treatment (12 weeks) and recovery periods was analysed. At necropsy, organs were examined grossly, weighed and prepared for microscopic evaluation. All gross lesions from any animal and all tissues from control and high-dose animals were examined. The following tissues were also examined: the mesenteric lymph node, spleen and thymus (both sexes), kidneys, forestomach and bone marrow from sternum (females) and adrenals (males) in the low- and mid-dose animals killed at the end of the treatment period and the control and high-dose animals killed at the end of the recovery period.

The concentrations of 5-CL and 5,7-DCL and metabolites were determined in plasma using LC-MS/MS. All plasma samples analysed from control animals were below the LLOQ (<9 ng/mL for 5-CL, <45 ng/mL for 5,7-DCL and <225 ng/mL for metabolites).

No 5-CL was quantifiable at 50 and 150 mg/kg bw per day. At 450 mg/kg bw per day, 5-CL was only quantifiable in week 13 at 1 hour after administration and only in two males and two females.

In general, there were no significant sex differences in plasma exposure after single or repeated dosing. After a single oral dose, C_{max} of 5,7-DCL and 5-chloro-8-hydroxyquinoline sulfate increased with dose so that they were quantifiable 1–3 hours post dosing. C_{max} for 5-chloro-8-hydroxyquinoline glucuronide and 5-chloro-8-hydroxyquinoline sulfate were achieved approximatively 1.2–2.1 hours post administration. 5-Chloro-8-hydroxyquinoline glucuronide was quantifiable in plasma from the first sampling time-point to 6 hours after a single dose, except in one male and one female in which levels were quantifiable from first sampling time-point to 24 hours. After repeated dosing, 5-chloro-8-hydroxyquinoline glucuronide was quantifiable 1 hour after administration at all dose levels. 5-Chloro-8-hydroxyquinoline sulfate was quantifiable in plasma 1 hour post dosing at 50 mg/kg bw per day for single and repeated dosing; 1–3 and 1 hour post dosing after single and repeated dosing, respectively, at 150 mg/kg bw per day; and from approximately 1–6 and 1 hour post dosing after single and repeated dosing, respectively, at 450 mg/kg bw per day. A moderate interanimal variability in plasma concentrations was observed; coefficient of variation ranged from 6% to 61% for 5-chloro-8-hydroxyquinoline glucuronide and from 9% to 53% for 5-chloro-8-hydroxyquinoline sulfate. No coefficient of variation was calculated for 5-CL due to the lack of quantifiable values.

Considering the moderate interanimal variability, exposures to 5,7-DCL, 5-chloro-8-hydroxyquinoline glucuronide, 5-chloro-8-hydroxyquinoline sulfate and 5,7-dichloro-8-hydroxyquinoline glucuronide increase approximately linearly with the dose of halquinol BP 80 administered on day 1. Exposure to 5,7-dichloro-8-hydroxyquinoline sulfate at the administered dose of 150 mg/kg bw per day in males was slightly lower than expected (assuming a similar dose to

AUC ratio as at the 50 and 450 mg/kg bw per day doses). These slight differences may be explained by the decreased dose during day 1 administration in males at 150 mg/kg bw per day and interanimal variability. No exposure data (AUC_{0-t}) were available for 5,7-dichloro-8-hydroxyquinoline sulfate at 1 hour in males at 150 mg/kg bw per day due to low concentration values.

No compound-related deaths occurred. At 450 mg/kg bw per day, the only adverse treatment-related clinical sign recorded was excessive salivation (ptyalism). No toxicologically significant effects were seen in functional observational battery, including motor activity, or ophthalmological examinations. Mean body weight, mean feed consumption and ophthalmological parameters were not affected. At 450 mg/kg bw per day, lower RBC count, haemoglobin concentration and haematocrit and higher mean urea and creatinine concentrations were observed in females; the haematology changes were associated with reduced extramedullary haematopoiesis noted in the spleen at 450 mg/kg bw per day. At the end of the treatment period (13 weeks), the concentration of total bilirubin was reduced at all doses in males (−46%, −8% and −24%, respectively, compared with control) and females (−30%, −28% and −19%, respectively). In females at all doses, decreases in the activities of ALP (−21%, −23%, −23%, respectively), aspartate transaminase (AST; −18%, −29% and −24%, respectively) and ALT; −8%, −26% and −26%, respectively) were observed; these changes were not associated with changes in liver histopathology. In males, ALP and ALT activities were reduced at 450 mg/kg bw per day (−24% and −11%, respectively). After the recovery period (17 weeks), total bilirubin was still decreased in males and females at 450 mg/kg bw per day (−57% and −13%, respectively); AST and ALT activities were also still decreased in females at 450 mg/kg bw per day. Dose-related increases in incidence of crystals in urine, which differed from that routinely observed, were seen in all treated groups. Both sexes at 450 mg/kg bw per day had a mean urine pH that was statistically significantly lower than controls; these findings correlated with increased urea and creatinine concentrations at clinical chemistry examination and with renal microscopic findings. After the 4-week recovery period, the urinary and clinical chemistry changes had reversed.

At necropsy, the caecum was distended with faeces in both sexes at 150 and 450 mg/kg bw per day, but there were no microscopic correlates. Organ weight changes consisted of decreases in mean heart weight at 150 mg/kg bw per day in females and 450 mg/kg bw per day in both sexes, but with no associated histopathological lesions; and minimal non-dose-related increases in mean adrenal weights in females at 50 mg/kg bw per day and above.

Halquinol-induced renal toxicity was characterized by microscopic lesions in females at 150 and 450 mg/kg bw per day; an increase in absolute and relative kidney weights in females at 150 mg/kg bw per day (+6% and +9%,

respectively, compared with control) and in males (+7% and +14%, respectively) and females (+34% and +42%, respectively) at 450 mg/kg bw per day; and macroscopic findings in one female at 450 mg/kg bw per day. At 450 mg/kg bw per day, all females showed a variable association of renal microscopic findings such as tubular basophilia/vacuolation and dilation, hypertrophy of distal tubules in the papillae, interstitial inflammation (mononuclear infiltrate) and occasional tubular degeneration/necrosis and granular casts. At 150 mg/kg bw per day, lower grade, unilateral, focal or multifocal renal microscopic findings seen in 6/10 females consisted of tubular basophilia/vacuolization (cortex), tubular dilation and hypertrophy of distal tubules in the renal papilla. These pathology findings in the high-dose group were not reversible over the 4-week recovery period.

In addition to the effects on kidneys, the following were noted: increased incidence of lymphoid atrophy in thymus in males at 150 mg/kg bw per day and in both sexes at 450 mg/kg bw per day; decreased development of the paracortex and germinal centres in the mesenteric lymph node at 450 mg/kg bw per day; and increased adrenal weights and cortical hypertrophy of adrenals at 450 mg/kg bw per day, which were suggestive of stress (according to the study director). These lymph node lesions were not observed in the control or lower dose groups in this study or at the highest dose (150 mg/kg bw per day) in the 1-year gavage rat study (Chevalier, 2017; section 2.2.3). Reduced extramedullary haematopoiesis noted in the spleen in both sexes at 450 mg/kg bw per day correlated in females only with decreased RBC count and haemoglobin concentration. At 450 mg/kg bw per day, an increase brown pigment in macrophages was observed in the mesenteric lymph node and a decrease in adipose tissue in the sternal bone marrow.

The NOAEL was 50 mg/kg bw per day based on increase in absolute and relative kidney weights and histopathological lesions in kidney in females at 150 and 450 mg/kg bw per day and in males at 450 mg/kg bw per day (Bentz, 2015a).

(b) Mini-pigs

In a GLP-compliant 28-day oral toxicity study, halquinol BP (98.82% purity; 26.2% 5-CL : 72.3% 5,7-DCL) in 0.5% w/v methylcellulose was administered to Göttingen mini-pigs (3/sex per group) by gavage at 0, 25, 75 or 225 mg/kg bw per day for 4 weeks at a constant dosage volume of 5 mL/kg bw. Body weight was recorded pretest and weekly during the study. Feed consumption was checked daily. Urine analysis and haematological and blood biochemical investigations were performed at the beginning and end of the study. Blood for the determination of halquinol BP 80 and its four main metabolites in plasma by LC-MS/MS was sampled in week 4 from surviving mini-pigs (non-GLP). At the end of the study the animals were killed under anaesthesia, necropsied and examined macroscopically. Selected organs were weighted. Histopathological

examination of male kidneys, liver, heart, skeletal muscle and seminal vesicles was conducted but not reported.

One animal at 225 mg/kg bw per day was euthanized in extremis with clear effects of toxicity (reduced feed intake, tremors, thin appearance, hypoactivity, hypothermia and liquid faeces). In the remaining animals, the following clinical pathology parameters were affected: urea, creatinine, ALT, AST, creatine kinase and lactate dehydrogenase (LDH). Body weights of females were not significantly reduced. Body weights of males at 225 mg/kg bw per day were reduced (−10% in mean variation from day 1). At necropsy, the absolute and relative kidney, liver, adrenal and thymus weights were increased at 225 mg/g per day in males and females. At 75 mg/kg bw per day, the absolute and relative kidney weights were statistically significantly increased in males (+22% and +4%, respectively, relative to control) and nonsignificantly increased in females (+7% and +8%, respectively). Gastrointestinal changes were seen in one animal at 225 mg/kg bw per day (red foci in the stomach and duodenum, white masses in the stomach and liquid green content in the caecum).

Although comparison in the absence of day 1 values is difficult, toxicokinetic analyses revealed that one of the two components (5-CL) was rapidly/entirely metabolized (below the limit of quantitation) and mainly into glucuronide for elimination.

A NOAEL was not determined because the histopathology findings were not reported. At 75 and 225 mg/kg bw per day, the only effect observed was a pronounced increase in absolute and relative kidney weights in males. No effects were seen at 25 mg/kg bw per day (Chevalier, 2014a).

In a 13-week oral toxicity study conducted in accordance with OECD TG 409 and International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products guideline (VICH GL43), Göttingen mini-pigs received vehicle (0.5% w/v of methylcellulose) or halquinol BP 80 (26% 5-CL : 72% 5,7-DCL) at 0, 25, 75 or 225 mg/kg bw per day (6, 4, 4 and 6 animals/sex per group, respectively) for 90 days. A constant dosage volume of 5 mL/kg bw was used. Although the study was conducted in accordance with GLP, the report does not contain the regulatory compliance statement (statement of quality assurance, pathologist report, etc.). For logistic reasons, the dosing of males and females was planned to start on two different days, 1 week apart. Dose levels were selected based on the results of the 28-day Chevalier (2014a) study.

Based on mortality and severe signs of toxicity in males at the high dose, treatment of all male groups ceased on day 3. To investigate the potential influence of the oral gavage probe used in the 28-day and the 13-week studies, six males previously in the control group were treated with halquinol BP 80 at 225 mg/kg bw per day using a 40 cm probe (Group 5; $n = 3$) or a 55 cm probe (Group 6; $n = 3$). Blood for the determination of plasma levels of halquinol BP 80 was

sampled on day 1. The concentrations of 5-CL and 5,7-DCL and their four main metabolites were determined in plasma using LC-MS/MS.

All animals were checked for mortality and morbidity at least twice a day and at least once a day for clinical signs. Detailed clinical examinations were performed, and body weights and feed consumption were recorded before treatment commencement and then once a week until the end of the study. Urine and blood were sampled for haematological and clinical chemistry analyses before treatment commencement, in week 6 and at the end of the treatment period. At the end of the study the animals were killed under anaesthesia, necropsied and macroscopically and microscopically examined.

Four out of six males at 225 mg/kg bw per day died after the second dose. Based on mortality and severe signs of toxicity in these high-dose males, treatment of all groups was stopped on day 3 (last dosing occurred on day 2) and dosing of females was not initiated. Treatment-related clinical signs such as soft/liquid and/or greenish colour faeces, hypoactivity, hypotonia, hypothermia and coldness to the touch were noted prior to death. These clinical signs were also observed at the mid dose of 75 mg/kg bw per day but at a lower incidence. The two surviving animals at 225 mg/kg bw per day showed changes in clinical pathology parameters (increased urea and creatinine levels and increased liver enzyme, creatine kinase and LDH activities). Histopathological evaluation identified severe acute tubular degeneration/necrosis of kidneys. Seminal vesicles in three animals showed marked sloughing of the epithelium lining of the tubulo-acini cells and was associated with marked to moderate oedema in the interstitium, with slight to moderate infiltrate of mixed inflammatory cells. Similarly, the prostate and urinary bladder showed slight or moderate oedema associated with infiltrate of mixed inflammatory cells in all three animals. Minimal to moderate single necrosis/apoptosis of hepatocytes was found in three out of four animals. In all four animals, glycogen was absent (in those animals found dead) or decreased (in animals euthanized in extremis). One animal (D70216) had acute focal degeneration/necrosis of the fundic mucosa of the stomach with haemorrhage and inflammation of the ileum, caecum, colon and rectum.

The toxicokinetic data show that on day 1 and at a T+2 hours, the glucuronide derived from 5-CL was the major metabolite measurable in plasma. The animal (D70221) euthanized on day 3 (at 225 mg/kg bw per day) had extremely high plasma values of the glucuronide forms. There was also moderate interanimal variability in the samples analysed. In addition, some values were out of range and were obtained by extrapolation. The values obtained on day 1 ($n = 6$) were similar to the values obtained on day 26 ($n = 2$).

5-Chloro-8-hydroxyquinoline, was completely and rapidly metabolized to 5-chloro-8-hydroxyquinoline glucuronide and 5-chloro-8-hydroxyquinoline sulfate; after 1 hour, it represented less than 1% of the metabolites while 5,7-DCL

was still detectable. As a result, plasma concentration of 5,7-DCL was calculated to be 10- to 25-fold lower in males than the plasma exposure of the metabolites. In females, 5,7-DCL exposure was approximately 5-fold lower than the plasma exposure of the metabolites.

To identify the reason for the significant difference in toxicity in the 28- and 90-day studies at the same dose levels, the procedural differences between the studies underwent detailed analysis. The applied dose levels were established to be correct, and stability was proven by re-analysis of dose formulations used in both studies. In both studies, toxicokinetic data confirmed that animals were exposed to the test item and its metabolites (mainly the glucuronide form). None of the environmental conditions were considered to have an impact on the studies. No differences in the animal batches were identified. The only obvious difference was the length of the tube used for gavage application; however, the follow-up investigation treating males at 225 mg/kg bw per day via the shorter probe tube resulted in similar signs of toxicity.

The inconsistent results from the two toxicity studies in mini-pigs despite the use of the identical test item, identical dose levels and comparable test conditions led to the conclusion that the mini-pig is not a suitable animal model for investigating oral toxicity of halquinol BP 80. The sponsor suggested that the stress of gavage treatment is a major contributor to the severity of and large interanimal variations in toxicity and toxicokinetic parameters specifically observed in the 90-day repeated-dose study. However, this interpretation does not take into account that the control and low-dose animals that were also treated by gavage did not show signs of stress such as adrenal or thymus alterations. It seems more likely that exposure to halquinol BP 80 increases susceptibility to stress.

Because of the unexplained differences in toxicity in the two studies, the use of mini-pigs as the non-rodent species for toxicity testing was abandoned in favour of the dog (Chevalier, 2014b,c).

(c) Dogs

In a GLP-compliant 13-week oral toxicity study conducted in accordance with OECD TG 409, El Amrani-Callens (2016a,b) administered halquinol BP 80 (26% 5-CL : 72% 5,7-DCL) to beagle dogs (4/sex per group) in gelatin capsules at doses of 0, 3, 10 or 60 mg/kg bw per day (Groups 1–4). An additional group (4/sex; Group 5) treated at 150 mg/kg bw per day was added approximately 1 month later due to the absence of any clinical signs or clinical pathology changes after 1 month of treatment at 60 mg/kg bw per day. Control animals received one empty capsule per day during the treatment period. Animals were caged individually and had free access to tap water and allowed approximately 220 g (per female) or

275 g (per male) pelleted feed per day. Following treatment initiation (including capsule gavage during the pretreatment period), appropriate treats (dog biscuits) were given to each animal for positive reinforcement. When necessary and after observation of the animals by a veterinarian, Group 5 (mainly females) received canned dog food according to their health status. From day 65, canned dog food was given when 15% of body-weight loss (from day −1) was observed. The animals in Group 5 were supplemented with one can of dog food per day from day 17 to 31. From day 31, feed was supplemented with 1–4 cans of dog food and up to 50 g biscuits in animals D5-1067 (days 32–43), D5-1068 (days 32–36 and 64–93), D5-1069 (days 32–55 and 57–69) and D5-1070 (days 32–50, 53–55, 57–63 and 71–93). Blood for the determination of plasma levels of halquinol BP 90 was sampled on day 1 and in week 13 from all surviving dogs, and on day 69 from one female (D5-1069) at 1, 2, 4, 6 and 24 hours post dose.

The dogs were checked at least twice daily for mortality and clinical signs. Detailed clinical examinations and body weight and feed consumption measurements of all animals were undertaken once before treatment initiation and once a week until study end. Ophthalmological examinations were performed before treatment initiation and at treatment end in control dogs and Groups 4 and 5 (60 and 150 mg/kg bw per day). Urine was collected in diuresis cages before treatment initiation in all the animals, on day 65 in Group 5 (females) and at treatment end in all animals (except for one animal, male D5-1020, in Group 5, from which urine was collected at necropsy). Blood samples for haematological and biochemical analyses were obtained before treatment initiation in all animals (Groups 1–4), on day 28 (Group 4 females) and day 35 (Group 4 males) and at treatment end in all surviving animals (Groups 1–4). In Group 5, blood was sampled at treatment initiation in all the animals, on day 21 (female D5-1069), on day 65 (all females) and at treatment end in all surviving animals. Haematological, blood biochemistry and urine analysis were performed in Groups 1–4 on days 0 (all animals), 28 (females) and 35 (males) and at treatment end; and in Group 5 on days 0 (all animals), 21 (female D5-1069) and 65 (females) and at treatment end.

All control and Groups 4 and 5 dogs were necropsied. Organs were examined grossly, designated organs weighed and selected tissue specimens preserved.

The concentrations of 5-CL and 5,7-DCL and their metabolites were determined in plasma by LC-MS/MS. All analysed plasma samples from control animals were below the LLOQ (<9 ng/mL for 5-CL, <45 ng/mL for 5,7-DCL and <225 ng/mL for metabolites). Following single or repeated oral dosing, T_{max} was approximately 1–2 hours after administration for 5-CL and its metabolites 5-chloro-8-hydroxyquinoline sulfate and 5-chloro-8-hydroxyquinoline β-D glucuronide. For 5,7-DCL and its metabolites 5,7-dichloro-8-hydroxyquinoline

sulfate and 5,7-dichloro-8-hydroxyquinoline β-D glucuronide, C_{max} was reached between 1 and 4 hours post dosing. When data were available, 5-chloro-8-hydroxyquinoline sulfate was the most abundant 5-CL metabolite at all dose levels except at 150 mg/kg bw per day, where 5-chloro-8-hydroxyquinoline β-D glucuronide and 5-chloro-8-hydroxyquinoline sulfate plasma exposure values were similar. A high interanimal variability was observed in thymidine kinase (TK) parameters for both 5-CL metabolites. No sex effect was observed for 5-CL, but insufficient data were available to perform such a comprehensive comparison. No pattern associated with multiple dosing was observed for 5-CL, 5-chloro-8-hydroxyquinoline β-D glucuronide or 5-chloro-8-hydroxyquinoline sulfate.

The exposure increased in an approximately linear manner with dose for 5-CL and its metabolites, but no definitive conclusions can be made due to the high interanimal variability.

One female at 150 mg/kg bw per day was euthanized prematurely on day 70, after vomiting, loss of appetite and pronounced body-weight loss. No other deaths occurred during the study although vomiting in males and females and inappetence in females was seen at 150 mg/kg bw per day; this was attributed to the test item, and these animals were isolated for at least 6 hours and given supplemented feed. Despite the supplemented feed, body-weight loss (up to −15%, compared with control, between days −1 and 94; $n = 3$) was observed in females at 150 mg/kg bw per day. There were no treatment-related ophthalmological findings in any animal. Males at 150 mg/kg bw per day had, compared with control, statistically significant decreases in reticulocyte count (−52%) as well as nonsignificant decreases in WBC (−20%), lymphocyte (−19%) counts; activated partial thromboplastin time (APTT; −31%), platelet count (−17%) and fibrinogen concentration (−14%) after 84 days of treatment. Females at 150 mg/kg bw per day had substantially significant reductions in WBC count (−21%) and nonsignificant decreases in reticulocyte and lymphocyte counts, fibrinogen concentration and APTT. Similar but less pronounced changes were observed at 60 mg/kg bw per day in males and females (e.g. decreases in WBC, RBC, reticulocyte and lymphocyte counts in females and decreases in reticulocyte, WBC, lymphocyte, APTT platelets and fibrinogen in males) after 85 days treatment. Similar effects were seen in the 39-week oral toxicity study in dogs by El Amrani-Callens (2017). At 150 mg/kg bw per day, blood biochemistry showed decreases compared with control (Group 1) values in ALP activities in males (−35%) and females (−19%) and in aspartate transaminase activity (−21%) in males only. At 150 mg/kg bw per day, cholesterol and triglyceride levels were increased in males and cholesterol levels only were increased in females. No effects were seen at urine analysis.

Group 5 (150 mg/kg bw per day) terminal body weights were lower, particularly in females, compared with controls. Mean absolute and relative

weights of liver and thymus were lower in males, reaching statistical significance for the absolute weight ($P < 0.05$ for both organs) and for the relative thymus weight ($P < 0.01$); this was mainly due to one individual value in each sex – male E51020 and female E51070. There were no treatment-related changes in the organ weights of animals treated at 3, 10 or 60 mg/kg bw per day (Groups 2–4).

Single cell necrosis along with focal necrosis and granulocytic infiltrate was observed in liver of one male at 60 mg/kg bw per day. At 150 mg/kg bw per day changes included more pronounced lymphoid atrophy in thymus; a trend in decreasing development of germinal centres in spleen; less glycogen in liver; and minimal diffuse acinar atrophy in parotid. No treatment-related histopathological changes were noted in the prematurely killed female at 150 mg/kg bw per day; a megaoesophagus was identified as the major cause of the poor health status of this animal. Atrophy of testes at 60 mg/kg bw per day (3 vs 1 in control) and hypospermatogenesis at 150 mg/kg bw per day were also observed.

In spleen, the development of germinal centre was less pronounced in males, particularly at 150 mg/kg bw per day. In kidneys, pigment was observed in the tubular epithelium of two males and one female at 150 mg/kg bw per day.

An overall NOAEL of 30 mg/kg bw per day was established based on lower terminal body weights at 60 mg/kg bw per day in males (El Amrani-Callens, 2016a,b).

In a GLP-compliant 39-week oral toxicity study conducted in accordance with OECD TG 409, El Amrani-Callens (2017) administered halquinol BP 80 (26% 5-CL : 72% 5,7-DCL) to beagle dogs (4/sex per group) in gelatin capsules as single daily doses of 0, 30, 60 or 90 mg/kg bw per day. Approximately 220 g (per female) or 275 g (per male) of pelleted diet were distributed daily. Following treatment (including capsule gavage during the pretreatment period), appropriate treats (dog biscuits) were given to each animal for positive reinforcement. Blood samples to determine the plasma concentrations of halquinol and its metabolites were collected on day 1 and in weeks 13 and 39 in all dogs at 1, 2, 4, 6 and 24 hours post dose. The plasma concentrations of halquinol and its metabolites were determined using LC-MS/MS.

Animals were observed daily for clinical signs. Feed consumption and body weight were measured weekly. Electrocardiography examinations (ECG) were performed on all animals before treatment initiation, and at least 2 hours after dose administration in control and high-dose animals once in weeks 12, 25 and 38. Low- and mid-dose groups also underwent ECG in week 39. Systolic and diastolic blood pressure values were recorded in control and high-dose females at the end of the treatment period. This was considered relevant only to females as there were no changes in ECG in males. Ophthalmological, haematological and clinical chemistry examinations were conducted before treatment initiation

and at weeks 12, 25 and 38 (termination). Urine analysis was conducted before treatment initiation and at weeks 12 (females), 13 (males), 25 and 38.

At the end of the treatment period, the animals were killed and a full macroscopic necropsy was performed. Tissues of all control and high-dose animals, kidneys of all animals and spleen and adrenal glands of low- and mid-dose animals were microscopically examined.

All plasma samples analysed from control animals were below the LLOQ (<9 ng/mL for 5-CL, <45 ng/mL for 5,7-DCL and <225 mg/mL for metabolites). T_{max} of 5-CL and its metabolites 5-chloro-8-hydroxyquinoline sulfate and 5-chloro-8-hydroxyquinoline β-D glucuronide was approximately 1–2 hours after single or repeated oral dosing with halquinol BP 80 in both sexes. T_{max} of 5,7-DCL and its metabolites 5,7-dichloro-8-hydroxyquinoline sulfate and 5,7-dichloro-8-hydroxyquinoline β-D glucuronide was between 1 and 4 hours. In general, no exposure data (AUC_{0-t}) were available for 5-CL. 5-Chloro-8-hydroxyquinoline sulfate and 5-chloro-8-hydroxyquinoline β-D glucuronide plasma exposure values were similar at all dose levels, with a slight but nonsignificantly higher exposure of 5-chloro-8-hydroxyquinoline β-D glucuronide. Plasma exposure of the sulfate metabolite of 5,7-DCL was higher than that of the parent compound and the glucuronide conjugate.

The results for each toxicokinetic parameter showed large intragroup variability; exposure values (relative to the parent and or its metabolites) increased for each administered compound in an approximately linear manner with the dose level of halquinol on day 1, week 13 and week 39. In general, no significant sex difference was observed in plasma exposure. After repeated-dose administration, the variability observed within each group was too large to be conclusive, but for 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, the data seems to consistently indicate a reduction of plasma exposure after repeated dosing.

No treatment-related ophthalmological and clinical effects were observed except for occasional hyperactivity in low- and mid-dose males and females. There were no deaths during the study. There were no significant treatment-related changes in mean body weight or mean body-weight change in any treated group; however, at the end of the treatment on week 38, a clear tendency towards a decrease in body weight in males was observed at 30 and 90 mg/kg bw per day (−7% and −6%, respectively, compared with controls) and in three females at 60 and 90 mg/kg bw per day (−10% and −15%, compared with controls). Urine analysis did not reveal significant findings at any dose level.

The ECG did not show significant findings at any dose level in males. Adverse ECG morphological abnormalities (decrease in heart rate, correlated with an increase in QT interval duration) were observed in one out of three

females at 90 mg/kg bw per day at treatment end. High QRS complex interval duration was observed in one out of four high-dose females at weeks 12, 25 and 38 (70, 78 and 78 ms, respectively, vs 42 ms at pretreatment). This correlated with ECG morphology changes (higher R wave amplitude [>3 mV] and negative T wave). No histopathological abnormalities were observed at microscopic examination of this animal's heart. A slight decrease in QT was observed at predose in mid-dose females (−9%, 189 ms vs 207 ms in control females at predose). There were no significant changes in diastolic or systolic arterial blood pressure in high-dose females in week 39. No investigations were performed in males as no ECG abnormalities were seen at examination.

The following haematological findings (all per cent changes are relative to control values) were observed at week 12 in high-dose males: decrease in RBC (−3%), basophil (−17%) and lymphocyte (−6%) counts and decrease in APTT (−20%) and fibrinogen (−11%) concentrations. Similar effects on WBC, RBC and basophil counts and fibrinogen and APTT were observed at 60 mg/kg bw per day. At week 25, decreases in lymphocyte count at 90 mg/kg bw per day and fibrinogen concentration were observed at 60 and 90 mg/kg bw per day. At week 38, decreases in WBC and basophil counts, fibrinogen concentration and APTT were observed at 60 and 90 mg/kg bw per day.

Changes relative to controls were as follows in females: at week 12, decreases in reticulocyte (−31%), basophil (−57%) and lymphocyte (−32%) counts and fibrinogen (−6%) concentration at 90 mg/kg bw per day, with similar effects at 60 mg/kg bw per day in basophil and lymphocyte counts and fibrinogen concentration. At week 25, decreases in WBC (−14%), platelet (−14%), reticulocyte (−15%) and lymphocyte (−24%) counts and fibrinogen concentration (−10%) at 90 mg/kg bw per day; similar effects were seen at 60 mg/kg bw per day in WBC and lymphocyte counts and fibrinogen concentration. At week 38, there was a decrease in basophil count (25%) at 90 mg/kg bw per day and in lymphocyte count (−16% and −25%, respectively) and fibrinogen concentration (−24% and −21%, respectively) at 60 and 90 mg/kg bw per day.

Haematological parameters were similarly altered in the 13-week oral toxicity study (El Amrani-Callens, 2016a,b) at day 85 (approximately 12 weeks of treatment). Therefore, the changes in haematological parameters in the dog at 60 mg/kg bw per day and higher doses were considered treatment-related findings.

An increase in cholesterol levels was observed in females at 60 mg/kg bw per day at week 25 (4.35 ± 1.08 vs 3.26 ± 0.10 nmol/L); 90 mg/kg bw per day at week 12 (3.63 vs 2.73 mmol/L; +33%); and both 60 and 90 mg/kg bw per day at week 38 (4.07 ± 1.03 vs 3.61 ± 1.2 and 3.86 ± 0.5 vs 3.61 ± 1.2, respectively). At week 25, LDH activity was significantly decreased ($P < 0.05$) in females at 90 mg/kg bw per day (76 ± 13.6 vs 131 ± 12.4 enzyme units [U]/L; −42%) and slightly decreased at 30 (−12%) and 60 (−16%) mg/kg bw per day. At week 38,

LDH activity was reduced at 60 (91 vs 120 U/L; −24%) and 90 mg/kg bw per day (84 ± 16.9 vs 120 ± 30.4 U/L; −30%). This reduced LDH activity was considered a treatment-related effect.

At weeks 12, 25 and 38, creatinine levels were slightly decreased in males at all doses. At week 38, urea concentrations in females were slightly decreased at all doses and creatinine levels at 90 mg/kg bw per day. These decreased urea and creatinine levels were associated with increases in kidney weight and histopathological effects at 90 mg/kg bw per day.

There were no significant findings in qualitative or quantitative urinary parameters at any dose. The only relevant finding was a decrease in urine volume at all doses in females: compared with controls (115 ± 32.5 mL), volumes were 37 ± 29.8 (−68%), 51 ± 40.8 (−56%) and 66 ± 33.1 (−43%) at 30, 60 or 90 mg/kg bw per day, respectively. This decrease in males was slight.

The only differences in organ weights were significant increases in the relative to body weights of liver (25.96 vs 40.74 g, 2.86% bw) and kidneys (32.23 vs 32.19 g, 0.4333% bw) in females at 90 mg/kg bw per day, probably due to decreases in body weight (−12.74% compared with control) at this dose level. A decrease in absolute and relative to body thymus weights in females was observed at all dose levels. At microscopic examination, treatment-related changes in the spleen included decreased development/no germinal centres at 90 mg/kg bw per day in males and females. Adrenal cortical hypertrophy was observed in one out of four mid-dose males, one out of four high-dose males and two out of four high-dose females. A low incidence and severity of decreased development of the spleen germinal centres was observed in mid-dose females. Papillary vacuolation/hypertrophy in the kidney urothelium and vacuolation of the inner cortex were seen in females at all doses. The urothelium papillary vacuolation/hypertrophy was similar in severity and incidence in the controls; however, vacuolation of the inner kidney cortex was elevated at high dose (2, 1 and 3 at, respectively, 30, 60 or 90 mg/kg bw per day, vs 1 for control). These findings were considered treatment-related based on the decrease in the volume of urine at all doses in females; this was associated with an increase in the relative kidney weights at higher doses and vacuolation of the inner kidney cortex at the highest dose level.

An overall NOAEL of 30 mg/kg bw per day was established based on lower terminal body weights in males at 60 mg/kg bw per day.

2.2.3 Long-term studies of toxicity and carcinogenicity

(a) Rats

In a GLP-compliant 1-year chronic toxicity study conducted in accordance with OECD TG 452, Chevalier (2017) administered halquinol BP 80 (26% 5-CL : 72% 5,7-DCL) in 0.5% w/v methylcellulose to Sprague Dawley rats (20/sex per group)

by gavage at doses of 0, 15, 50 or 150 mg/kg bw per day. A constant dosage volume of 5 mL/kg bw was used in all groups. Toxicokinetic evaluations were carried out in the 4-week and 13-week toxicity studies (Bentz, 2015a; Chevalier, 2015), and no further toxicokinetic investigations were conducted in the present study.

Assessment of toxicity was based on mortality, clinical observations, body weights, feed consumption and ophthalmological (pretest and at week 51), haematological, clinical pathology (weeks 13, 26 and 52) and anatomical pathology evaluations (including organ weights). Body weights, feed consumption, clinical signs and incidence/location of tissue masses were determined weekly for the first 13 weeks and monthly thereafter. At study termination, the animals were killed and a full macroscopic examination was performed. Organs were weighed and tissues were collected, preserved and microscopically evaluated. All tissues from animals in the control and high-dose groups or that died or were euthanized, or from tissues with macroscopic abnormalities were histopathologically examined. In addition, kidneys (females), liver (males) and mammary glands (females) of animals at 15 or 50 mg/kg bw per day were examined. Sperm and testes were sampled at study end to clarify the findings in the Spezia (2016) two-generation reproduction study (section 2.2.5(a)). Animals were observed daily for mortality and moribundity.

There were no treatment-related deaths, toxicologically significant clinical signs, changes in body weight or feed consumption or ophthalmological effects. Haematological parameters such as reticulocyte count (% and total cells/L) and monocytes were decreased at all doses in males at 26 and 52 weeks. This effect was neither dose related nor consistently seen in both sexes. In addition, WBC, lymphocyte and neutrophil counts were decreased at all doses in males at 52 weeks. In females at 52 weeks, a decrease in WBC, platelet, reticulocyte, eosinophil, lymphocyte and monocyte counts was observed at all doses and in neutrophil count at 50 and 150 mg/kg bw per day. Haematological effects were not dose related, consistent throughout the study or observed in both sexes. In addition, no changes in extramedullary haematopoiesis in the spleen were reported. Therefore, the haematological effects were not considered adverse or toxicologically significant. Total bilirubin concentration was decreased at weeks 26 and 52 in males at 50 and 150 mg/kg bw per day and in females at all doses. ALP, ALT and AST activities were decreased at all doses in males and females at 52 weeks. However, histopathological liver changes were observed only in males, particularly at 150 mg/kg bw per day. No effects on seminology parameters were detected. Urine analysis found no alterations throughout the treatment period except for a slight decrease (−18%) in urine volume at week 26 in females in all treatment groups.

At necropsy, caeca of animals at 150 mg/kg bw per day, but particularly males, had basophilia/vacuolation dilated with faeces. Although the mean

absolute spleen weight was statistically significantly higher in females at 15 mg/kg bw per day ($P < 0.05$), this difference was minimal (15%) and there was no dose-related trend, excluding any relationship with the test item.

The mean absolute and relative kidney weights were statistically significantly higher in females at 150 mg/kg bw per day ($P < 0.01$), correlating with microscopic changes. In females at 150 mg/kg bw per day alone, compared with control, kidneys showed minimal to moderate focal/multifocal basophilia/vacuolation and dilatation (13/17 vs 0/19); hypertrophy of distal tubules in papilla (6/17 vs 0/19) along with mononuclear cell inflammatory cells (11/17); and occasionally degeneration/necrosis of tubules (5/5). In the most affected areas, tubules had a thickening of the basement membrane and fibrosis with deformation (depression) of the surface. Similar changes such as basophilia and vacuolization of the tubules (3/19) and hypertrophy of distal tubules in papilla (1/17) without degeneration/necrosis of tubules, were observed in females at 50 mg/kg bw per day. Minimal tubular dilation was seen in one female at 15 mg/kg bw per day. In the absence of other treatment-related changes in kidneys of this animal, this isolated change was considered incidental and not treatment related.

These changes correlated with kidney deformations found at necropsy in a few animals at 150 mg/kg bw per day and with minimal higher absolute and relative kidney weights (up to +16%). Degeneration/necrosis of epithelial cells indicated that this dose level was adverse. The Committee also considered the slight kidney changes (focal/multifocal basophilia/vacuolation) in females at 50 mg/kg bw per day to be treatment-related. No treatment-related changes were noted in kidneys of males.

In liver of males only, there was a trend towards a slightly higher severity of vacuolation (with a midzonal distribution) in all treated males, particularly at 150 mg/kg bw per day (20/11, 19/9, 19/14) compared to controls (17/7). In the pancreas of males, there was higher incidence in fibrosis/haemosiderosis at 150 mg/kg bw per day compared to controls (11/17 vs 16/19).

There was a trend towards a slightly higher incidence and severity of dilation of mammary gland ducts in high-dose females, but this was considered unrelated to treatment as this is commonly seen in female test animals. However, the incidence of mammary gland adenocarcinoma in high-dose females was higher than controls (4/17 vs 2/19). The incidence of benign fibroadenomas was similar in treated and control animals.

The NOAEL was 15 mg/kg bw per day based on changes in kidney histopathology associated with an increase in mean absolute and relative kidney weights in females at 50 mg/kg bw per day (Chevalier, 2017).

Table 1
Summary of genotoxicity assays on halquinol BP 80

Study type	Test system	Concentration / dose tested	Results	Reference
In vitro				
Ames test	*Salmonella typhimurium* TA98, TA100	5,7-DCL at 0.05 g/mL (±S9) [a]	Negative (±S9)	Nagao et al. (1977)
Miniscreen Ames test	*S. typhimurium* TA98, TA100	5-chloro-8-hydroxyquinoline sulfate [b] 5-chloro-8-hydroxyquinoline glucuronide [c] 5,7-dichloro-8-hydroxyquinoline sulfate [d] 5,7-dichloro-8-hydroxyquinoline glucuronide [e] At 5, 15, 50, 150, 500 µg/well (±S9)	Negative (±S9)	Frieauff (2013a,b,c,d)
Gene mutations	L5178Y $tk^{+/-}$ mouse lymphoma cells	0.31, 0.63, 1.25, 2.5, 5, 10, 20 and 40 µg/mL (±S9; 40 µg/mL precipitated)	Positive (±S9)	Sire (2014b)
Chromosomal aberration	Human lymphocytes	0, 0.78, 1.56, 3.13, 6.25, 12.5, 25, 50 and 100 µg/mL (−S9) 0, 3.13, 6.25, 12.5, 18.8, 25, 37.5, 50 and 100 µg/mL (+S9)	Positive (+S9)	Sire (2014c)
In vivo				
Micronucleus induction	Rat	500, 1 000 and 2 000 mg/kg bw per day (oral)	Negative	Sire (2014a)
Chromosomal aberration	Rat	500, 1 000 and 2 000 mg/kg bw per day (oral)	Negative	Ciliutti (2014)

bw: body weight; S9: 9000 × *g* supernatant fraction from liver homogenate from induced rats (metabolic activation)
[a] Bacteriotoxicity was observed at ≥150 µg/well using strains TA98 and TA100 (+S9) and at 500 µg/well using strain TA100 (−S9).
[b] Bacteriotoxicity or precipitation was observed at ≥150 µg/well using strains TA98 and TA100 (±S9).
[c] Neither precipitation and nor bacteriotoxicity was observed using TA98 and TA100 (±S9).
[d] Precipitation was noted at 500 µg/well using TA98 and TA100 (+S9). Bacteriotoxicity was observed at 500 µg/well using TA98 and TA100 (−S9).
[e] Precipitation was observed at 150 µg/well using TA98 and TA100 (+S9) and at 500 µg/well using the strain TA100 (−S9).

2.2.4 Genotoxicity

Five studies (in vitro and in vivo) were performed to examine the genotoxicity potential of halquinol BP 80. The results are summarized in Table 1.

A non-GLP-compliant bacterial reverse mutation test examined the ability of 5,7-DCL to induce mutations in *Salmonella typhimurium* strains TA100 and TA98 (frame-shift mutation-sensitive strains). The results indicated that 5,7-DCL was negative in two strains of *S. typhimurium* with and without S9 mix at relatively low concentrations (0.05 µg/mL) (Nagao et al., 1977).

Non-GLP-compliant bacterial reverse mutation tests were conducted to examine the ability of the major metabolites (5,7-dichloro-8-hydroxyquinoline glucuronide, 5,7-dichloro-8-hydroxyquinoline sulfate, 5-chloro-8-hydroxyquinoline glucuronide and 5-chloro-8-hydroxyquinoline sulfate) to induce mutations in *S. typhimurium* strains TA98 and TA100 at concentrations of 5, 15, 50, 150, 500 µg/well with and without metabolic activation (S9 mix). Dimethylsulfoxide (DMSO) was used as the solvent.

There were no relevant increases in revertants over the corresponding negative control values with any of the tester strains with or without metabolic

activation in either the plate incorporation assay or the preincubation assay. It was concluded that the test articles did not show evidence of a mutagenic potential in the bacterial mutation assay under the conditions of the test (Frieauff, 2013a,b,c,d).

Non-GLP-compliant TK6 in vitro micronucleus tests were conducted to examine the clastogenic and/or aneugenic potential of the major metabolites (5,7-dichloro-8-hydroxyquinoline glucuronide, 5,7-dichloro-8-hydroxyquinoline sulfate, 5-chloro-8-hydroxyquinoline glucuronide and 5-chloro-8-hydroxyquinoline sulfate) at concentrations from 11 to 500 µg/mL with and without metabolic activation system (S9 mix). DMSO was used as the solvent.

It was concluded that the major metabolites were neither clastogenic nor aneugenic under the conditions of the test (Frieauff, 2014a,b,c,d).

In a GLP-compliant study conducted in accordance with OECD TG 476 and VICH GL23, Sire (2014b) tested halquinol BP 80 (26.29% 5-CL : 72.53% 5,7-DCL) for its potential to induce mutations in an in vitro mammalian cell gene mutation test using L5178Y $tk^{+/-}$ mouse lymphoma cells. After a preliminary toxicity test (see section 2.2.1(b)), halquinol BP 80 dissolved in DMSO was tested in a single main experiment, with and without metabolic activation (S9 mix). Cultures of 5×10^5 cells/mL were exposed to the test substance or vehicle and positive controls in the presence or absence of S9 mix (final concentration of S9 fraction 2%) for 3 hours. Cytotoxicity was measured by assessing adjusted relative total growth, adjusted relative suspension growth and cloning efficiency following the expression time. The number of mutant clones (differentiating small and large colonies) was evaluated after expression of the mutant phenotype. Selection of the highest dose level for the main experiment was based on the level of toxicity, according to the criteria specified in the OECD guidelines (decrease in adjusted relative total growth). The selected dose levels were 0.31, 0.63, 1.25, 2.5, 5, 10, 20 and 40 µg/mL, with and without S9 mix. At the end of the treatment period, a precipitate was noted in the culture medium at 40 µg/mL.

Without S9 mix, moderate to severe toxicity was induced at 5 µg/mL and higher doses, as shown by a 53–100% decrease in the adjusted relative total growth. Dose-related increases in mutation frequency exceeded the global evaluation factor of 126×10^{-6} at dose levels of 5, 10 and 20 µg/mL, inducing acceptable levels of cytotoxicity; these results met the criteria of a positive response. With S9 mix, marked severe toxicity was induced at 2.5 µg/mL and higher doses, as shown by a 64–100% decrease in the adjusted relative total growth. Dose-related increases in mutation frequency exceeded the global evaluation factor at 2.5 and 5 µg/mL, inducing acceptable levels of cytotoxicity; these results met the criteria of a positive response.

Under the conditions of this study, halquinol BP 80 showed a mutagenic activity in L5178Y $tk^{+/-}$ mouse lymphoma assay, both in the presence and absence of S9 (with and without metabolic activation) (Sire, 2014b).

In a GLP-compliant study conducted in accordance with OECD TG 476 and VICH GL23, Sire (2014c) tested halquinol BP 80 (26.29% 5-CL : 72.53% 5,7-DCL) for its potential to induce chromosomal aberrations in cultured human peripheral blood lymphocytes with and without metabolic activation.

Two experiments were conducted with halquinol using DMSO as the solvent, both with and without S9 mix. The highest dose level for treatment in the first experiment was selected based on solubility data. For selection of the dose levels for the second experiment, any toxicity indicated by the reduction of mitotic index in the first experiment was also considered. For each culture, mitogen (phytohaemagglutinin)-stimulated cells were incubated with halquinol or control substance, with or without S9 mix, and subsequently subjected to metaphase arrest (using Colcemid® solution). Positive controls were methylmethane sulfonate (–S9) and cyclophosphamide (+S9).

Eight doses of the test article, ranging from 0 to 800 µg/mL (with and without S9; 20-hour treatment; 0-hour recovery) were used in the first experiment, and eight doses ranging from 0 to 100 µg/mL (with S9, 3-hour treatment, 17-hour recovery) and without S9 (44-hour treatment, 0-hour recovery) were used in the second experiment. In the experiments without S9 mix, after a 3-hour treatment a 48–100% decrease in mitotic index was observed at 25 µg/mL and higher dose levels (first experiment). Following the 20-hour treatment (second experiment), an 86–100% decrease in mitotic index was noted at 25 µg/mL and higher dose levels. Following the 44-hour treatment (second experiment), a 49–100% decrease in mitotic index was noted at dose levels 6.25 µg/mL and higher dose levels.

No significant increase in the frequency of cells with structural chromosomal aberrations was noted after the 3- or 44-hour treatments. An increase in the frequency of cells with chromosomal aberrations was noted following the 20-hour treatment with the highest analysed dose level, 12.5 µg/mL. This increase was statistically significant and exceeded the historical control data for the vehicle control ($P < 0.05$; 4% vs 0–2.5% for the historical control data). This increase was not tested for its reproducibility; thus, in the absence of any similar results following the 3- or 44-hour treatments, it remained equivocal.

In the experiments with S9 mix, a 38–100% decrease in mitotic index was observed at 25 µg/mL and higher dose levels in the first experiment. In the second experiment, 44–100% decreases in mitotic index were noted at 18.8 µg/mL and higher dose levels.

Statistically significant increases in the frequency of cells with structural chromosomal aberrations were noted at 25 µg/mL (6.9%; $P < 0.001$) in the first

experiment and at the same dose level (5.5%; $P < 0.01$) in the second experiment. These results met the criteria of a positive response.

Under the conditions of this study, halquinol BP 80 induced chromosomal aberrations in cultured human lymphocytes in the presence of S9 (metabolic activation) (Sire, 2014c).

In a GLP-compliant study conducted in accordance with OECD TG 475, Sire (2014a) tested halquinol BP 80 (26.29% 5-CL : 72.53% 5,7-DCL) for its potential to induce damage to the chromosomal or the mitotic apparatus in rat bone marrow cells. Male Sprague Dawley rats (8–9 weeks old; 5/group) received a single oral dose of halquinol BP 80 in 0.5% methylcellulose in deionized water at 0, 500, 1000 or 2000 mg/kg bw. The doses were selected based on a preliminary toxicity test. An additional group was dosed orally with 15 mg/kg bw of the positive control item, cyclophosphamide. For the evaluation of the plasma levels of halquinol, satellite groups (3/sex) were given halquinol at the highest dose.

LC-MS/MS was used to determine the concentrations of the test item compounds (5-CL and 5,7-DCL) and their respective 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) in plasma samples from animals given 2000 mg/kg bw per day, 1 and 6 hours after the single treatment. The concentrations of the test item and its metabolites in plasma samples demonstrated that the bone marrow of the treated animals was adequately exposed (representative nominal concentration for 5-CL in plasma: 24.5, 147 and 610 ng/mL for the 27, 150 and 600 ng/mL).

The treated and vehicle control animals were killed 24 or 48 hours after treatment and the positive control animals were killed 24 hours after treatment. Bone marrow smears were then prepared and scored. The number of the micronucleated polychromatic erythrocytes (MPE) was counted per 2000 polychromatic erythrocytes (PE). The polychromatic erythrocyte (PE) to normochromatic erythrocyte (NE) ratio was established by scoring a total of 1000 erythrocytes (PE + NE). A complementary analysis of 2000 supplementary cells per animal was undertaken in males only. The PE : NE ratio in the halquinol-treated groups did not differ significantly from that in the vehicle control group.

A slight increase in the number of micronucleated cells was observed only in males at 2000 mg/kg bw. The frequencies of micronucleated cells were, however, well within the historical vehicle control data range (2.2 and 1.9 MPE per 1000 PE, for the 24- and 48-hour sampling times, respectively, versus 0.3–2.7 MPE per 1000 PE for the historical control data). The slight increase in micronucleated cells in males was therefore considered not biologically relevant, and the study results were considered a negative response.

Under the experimental conditions of the study, 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 (Sire, 2014a).

In a GLP-compliant study, Ciliutti (2014) investigated the potential of halquinol BP 80 (26% 5-CL : 72% 5,7-DCL) to induce chromosomal damage in rat bone marrow cells in vivo. In the main study, four groups (5/sex per group) received a single oral administration of 0 (0.5% methylcellulose vehicle), 500, 1000 or 2000 mg/kg bw. An additional group of animals was dosed once by intraperitoneal route with the positive control item, mitomycin C, and sampled at 18 hours. An additional five animals each were included in the control and high-dose groups for sampling time at 42 hours.

No clinical signs were observed in any treatment group. The animals were injected intraperitoneally with colchicine (4 mg/kg bw) and killed. Bone marrow cell suspensions were prepared on slides subsequently stained.

Halquinol BP 80 did not induce remarkable inhibitory effects on the bone marrow cells at any dose level as indicated by mitotic index results. Following treatment with halquinol BP 80, a slight reduction of the mitotic index (approximately 27% and 31%, respectively) was observed for the high- and mid-dose groups at the 18-hour sampling time. No reduction was observed at the 42-hour sampling time. The sponsor noted, however, that at this sampling time two animals (nos 64 and 66) showed a very low number of metaphases, possibly due to colchicine misdosing or an oversight during preparation of cell suspension, and were excluded from the mitotic index evaluation.

Following treatment with halquinol BP 80, a slight increase (0.0–0.4%) in the incidence of aberrant cells was observed in the treated groups. Statistically significant increases in the incidence of aberrant cells over the control values were observed following treatment with the positive control mitomycin C, indicating the validity of the test system.

Under these experimental conditions, oral administration of halquinol BP 80 does not induce chromosomal aberrations in rat bone marrow cells treated to a limit dose of 2000 mg/kg bw (Ciliutti, 2014).

The USFDA evaluated halquinol for genetic toxicity via (quantitative) structure–activity relationship [(Q)SAR] models using three software programs, Derek Nexus 5.0.2 (DX), Leadscope Model Applier 2.2.2–3 (LMA) and CASE Ultra 1.6.2.3 (CU). The evaluation revealed the presence of structural alerts for mutagenicity and clastogenicity for all three components of halquinol (5,7-DCL, 5-CL and 7-CL).

The positive clastogenicity observed in vitro was not confirmed in vivo. However, the positive result in the mammalian mutagenicity study in vitro was not addressed by the in vivo studies (micronucleus induction and chromosomal aberration), both of which were performed using bone marrow, because they do not assess the induction of a mutation.

In the absence of a carcinogenicity study or an in vivo gene mutation study, the Committee was unable to reach a definitive conclusion with respect to the genotoxic potential of halquinol.

2.2.5 Reproductive and developmental toxicity studies

a) Multigeneration reproduction toxicity

In a GLP-compliant two-generation reproductive toxicity study conducted in accordance with OECD TG 416, Spezia (2016) administered halquinol BP 80 (26% 5-CL : 72% 5,7-DCL) to Sprague Dawley rats (24/sex per group) by gavage at 50, 150 or 450 mg/kg bw per day for 10 weeks prior to and during pairing, through gestation and lactation until weaning. Control groups received the vehicle (0.5% w/v methylcellulose in drinking-water). Dose selection was based on results from a previous dose range–finding study (Spezia, 2015a,b). The halquinol BP 80 concentrations in the administered dose formulations analysed in the first and last days of the treatment period remained within an acceptable range of variations (−5.2% to −0.3%) compared to the nominal values (±15% of the nominal concentrations).

On postnatal day 22, one or two F_1 males and one or two F_1 females per litter were selected to mate and litter (24 animals/sex per group). The F_1 rats received the test item daily, via gavage, at a constant dosage volume of 5 mL/kg bw, for 10 weeks prior to pairing, during pairing, and through gestation and lactation until weaning of the F_2 pups (postnatal day 21).

Clinical signs and mortality in all generations were checked daily. Female body weight and feed consumption were measured once a week, over a 7-day period, from the first day of treatment until the start of the mating period, on gestation days 0, 7–14 and 14–20 and during lactation on postnatal days 1–7, 7–14 and 14–21. Male body weight and feed consumption were recorded on the first day of treatment (study day 1), then once a week until scheduled death. During the mating period, feed consumption was not measured for males or females.

F_0 and F_1 males and females were paired until mating or after 14 days had elapsed. The females were allowed to deliver normally and rear their progeny. Pregnancy and litter parameters were recorded. During lactation, the F_1 and F_2 pups were observed daily for survival and clinical signs. The morning when parturition was completed was designated day 1 and each pup was identified. Total litter size and sex of each pup were recorded as soon as possible after birth. Any gross external malformations in pups were noted. The litters were observed daily.

On postnatal day 4 the size of each litter was adjusted by randomly culling pups to obtain, as near as possible, four/sex per litter. The pups were observed daily for clinical signs, abnormal behaviour and external abnormalities.

The weight of each pup was recorded on postnatal days 1, 4, 7, 14 and 21. The sex ratio was recorded. The following physical and/or reflex development parameters were assessed: pinna unfolding and hair growth on postnatal day 5; tooth eruption on postnatal day 13; auditory canal and eye opening on postnatal day 17; surface righting reflex on postnatal day 5; cliff avoidance on postnatal day 11; and air righting reflex on postnatal day 17. After weaning, F_0 and F_1 parents were anaesthetized and killed by exsanguination. Designated organs were weighed in parents and offspring (1 pup/litter). Epididymal and testicular sperm parameters were evaluated in F_0 and F_1 males. All F_0 and F_1 parents and on one F_1 and F_2 pup per sex and per litter killed on weaning underwent macroscopic examination. Any pups that died or were killed prematurely during the lactation period also underwent macroscopic examination. Macroscopic lesions, reproductive organs, adrenals, brain, kidneys, liver, spleen, thyroids and pituitary glands were sampled in all parent animals. Macroscopic lesions and reproductive organs of all F_0 and F_1 parents of the control and high-dose groups were microscopically examined.

Three F_0 animals were found dead: one low-dose male, one high-dose male and one high-dose female. One high-dose female was euthanized on postnatal day 1 as its litter was dead. Previously, this female had piloerection, round back, pale extremities and chromorhinorrhoea. Animals found dead or euthanized in poor condition were not histopathologically examined.

Ptyalism was the most distinct clinical sign observed in males and females at 450 mg/kg bw per day throughout premating, mating, pregnancy and lactation. There were no adverse effects on pre- and post-mating mean body weight or mean body-weight changes. However, there were significant reductions in mean body weight and mean body-weight changes during pregnancy (prenatal days 7, 14 and 20) at 450 mg/kg bw per day. During lactation, mean body weight at 450 mg/kg bw per day was significantly reduced compared with control on postnatal day 1; however, this reduction was minimal from postnatal day 4 to 21. There were no treatment-related effects on feed consumption before mating (males and females) or after mating (males) or during gestation (females) and lactation. There were no treatment-related effects on estrous cycles, mating, fertility and delivery.

The mean testicular sperm heads (10^6 per g testis) in F_0 males were reduced at all doses compared with concurrent control (130.3 ± 16.3) but were within the range provided for historical controls (121.4–134.4). The testicular sperm head sperm count was decreased at week 18 at 50 mg/kg bw per day; this was attributed to one male (E22630) and considered coincidental. No other sperm parameters were affected. In F_0 parents, a statistically significantly higher mean relative kidney weight were seen in males at 150 mg/kg bw per day ($P \leq 0.01$). Absolute and relative kidney weights were higher in both sexes at 450 mg/kg bw per day; these effects were considered treatment-related.

The mean absolute and relative spleen weights were higher in males at all dose levels, but particularly at 150 and 450 mg/kg bw per day. Statistical significance ($P \leq 0.05$ and ≤ 0.01, respectively) for the relative weight was reached at 150 and 450 mg/kg bw per day. The mean relative liver weight was higher in males at 450 mg/kg bw per day (+9%; $P \leq 0.05$) and the mean absolute liver weight was lower in females at 150 mg/kg bw per day (–9%; $P \leq 0.05$).

A slight but non-dose-related increase in incidence of dilated pelvis in kidneys was observed at all doses ($n = 2, 1, 1$, respectively) in females. As no other histopathological effects were observed, this effect was not considered adverse or biologically relevant. Degenerative corpora lutea with inflammatory cells was observed at a slightly higher incidence in females at 450 mg/kg bw per day but there no increase in severity compared to controls. No treatment-related changes were seen in the male sex organs.

In the F_0 offspring, no treatment-related deaths or effects on viability and lactation indexes were observed. No clinical effects were noted during lactation. Mean body weight and mean body-weight changes were unaffected in pups. There were no effects on pup physical and reflex development landmarks.

In F_1 animals, there were no treatment-related deaths. Ptyalism was observed in mid-dose (4 vs 0) and high-dose (19 vs 0) males and in females (during the pre-mating and mating periods). High-dose females showed ptyalism during pregnancy and lactation. There were no treatment-related adverse effects on mean body weight except for a decrease in weight in high-dose males; decreases in mean body-weight changes occurred in all the treated groups from day 106 to 113, reaching statistical significance from day 113 to 120. In females at 450 mg/kg bw per day, the reduction in body weight at days 7, 14 and 20 was associated with a statistically nonsignificant reduction in body-weight gain from days 0 to 20. Mean feed consumption was significantly decreased in pregnant females at 50 and 150 mg/kg bw per day from day 14 to 20 and slightly nonsignificantly reduced at 450 mg/kg bw per day. The mean age of cleavage of balanopreputial gland or vaginal opening was not affected. At 4 weeks of age, all pups had a positive pupil constriction reflex. There were no toxicologically significant effects on motor activity evaluation and no treatment-related effects on auditory startle reflex test. The estrous cycle in F_1 females was comparable in control and treated animals. From 150 mg/kg bw per day, and compared with controls, mean number of epididymal sperm cells (per cauda or per g of cauda) was lower than in control and below the lower limit of historical control data. Epididymal non-motile sperm values increased at mid and high dose (143% and 200%, respectively, compared with controls). In F_1 parents, the mean relative kidney weights were statistically significantly higher in males from mid- and high-dose parents ($P \leq 0.01$ and ≤ 0.05, respectively). The absolute and relative kidney weights of females from high-dose parents were higher ($P \leq 0.01$). F_1 male

parents at 450 mg/kg bw per day group have slightly higher incidence of kidney tubular basophilia (23/3 vs 23/1 in control). Degenerative (large) corpora lutea with inflammatory cells were observed at a slightly higher incidence in high-dose F_1 female parents (7/24 vs control 1/22). Some vacuolization of testes and inflammation of prostate was observed in high-dose F_1 male parents. In the F_1 offspring, there were no treatment-related deaths or effects on viability and lactation indexes. The incidence of kidney tubular basophilia was higher in high-dose F_2 female pups (24/19 vs control 18/13) and similar in males. No clinical effects were noted during lactation. Mean pup body weight and mean body-weight changes were unaffected. There were no effects on anogenital distance, pup physical and reflex development landmarks.

The NOAEL for parental toxicity was 50 mg/kg bw per day based on increased kidney weights at 150 mg/kg bw per day and above in F_0 and F_1 parents.

The NOAEL for offspring toxicity was 150 mg/kg bw per day based on changes in kidney histopathology in pups at 450 mg/kg bw per day.

The NOAEL for reproductive toxicity was 450 mg/kg bw per day based on the absence of any treatment-related effects (Spezia, 2016).

b) Developmental toxicity

Mice

In a dose range–finding study reported to be non-GLP-compliant, time-mated female mice (10/group, except 4 in the highest dose group) were dosed orally by gavage with halquinol (26% 5-CL : 72% 5,7-DCL) at 100, 300 or 1000 mg/kg bw per day during the period of organogenesis (from implantation to closure of the hard palate: gestation days 6–15, inclusive). A constant dose volume of 5 mL/kg bw was used. Two females at 1000 mg/kg bw per day died and the remaining two were prematurely killed on gestation day 13 because the maximum tolerated dose was exceeded. Another group of previously untreated mice (Group 5, $n = 7$) was administered 600 mg/kg bw per day.

All animals were checked daily for mortality and clinical signs. Body weight and feed consumption were evaluated on gestation days 3, 6, 9, 12, 16 and 18 and prior to any unscheduled kills. Females were killed on gestation day 18 and macroscopically examined. Numbers and distributions of implantation sites, early and late resorptions, and live and dead fetuses were recorded. The fetuses were sexed, weighed and examined for external abnormalities.

At scheduled kill, round back, piloerection, dyspnoea, reddish vaginal discharge and/or emaciated appearance were observed in one female at each dose level. The following effects were seen at 600 mg/kg bw per day: lower body weight with no effects on feed consumption; lower gravid uterus; and lower fetal body weights. There were statistically significant dose-related increases in mean

postimplantation losses at 100 (18.2%), 300 (31.9%) and 600 (47.8%) mg/kg bw per day compared to control (4.0%). At 300 mg/kg bw per day, there were two malformed fetuses (exencephaly or meningocele) in one litter. As a result, 300 mg/kg bw per day was chosen as a suitable high dose for the developmental toxicity study (Papineau, 2015).

In a GLP-compliant study conducted in accordance with OECD TG 414 and VICH GL32, Spezia (2015a,b) examined the potential for developmental toxicity of halquinol BP 80 in mice. Halquinol BP 80 (26% 5-CL : 72% 5,7-DCL) in 0.5% w/v methylcellulose was administered to time-mated Swiss:RjOrl mice (24/group) by gavage at doses of 0, 30, 100 or 300 mg/kg bw per day over gestation days 6–17 inclusive (the organogenesis period). A constant dosing volume of 5 mL/kg bw was used in all the animals.

The animals were checked daily for mortality and clinical signs. Body weights and feed consumption were recorded every 2 or 3 days throughout the study. On gestation day 18, the females were killed and macroscopically examined. The fetuses were removed by caesarean section; the numbers of implantations, uterine scars, early and late resorptions and live and dead fetuses were recorded; and the fetuses were weighed, sexed and examined for external, soft tissue and skeletal abnormalities.

There were no effects on pregnancy status. At scheduled kill on gestation day 21, 16/24, 21/24, 17/24 and 14/24 females at 0, 30, 100 and 300 mg/kg bw per day, respectively, had live fetuses. There were two deaths at the mid dose and one at the high dose, for which a treatment-related effect cannot be excluded despite the absence of clinical signs prior to death and/or findings noted at necropsy. Treatment-related effects at mid and higher doses included round back, piloerection, pale extremities, emaciated appearance and/or hyperactivity/hypoactivity. At 300 mg/kg bw per day, mean body weight was lower (not statistically significantly), resulting in a lower mean body-weight change; this finding was considered treatment related. Feed consumption was not affected. There were no effects on mean carcass weight and net body-weight change. The dose-related decrease in mean gravid uterus weight seen at all doses achieved statistical significance at 300 mg/kg bw per day (−24% compared with control). Considering the amplitude of the changes, this finding was toxicologically significant at all doses (−10%, −12% and −24% at 30, 100 and 300 mg/kg bw per day, respectively, compared to control). Similarly, the mean number of live fetuses per female was decreased at 100 (n = 11.9) and 300 (n = 8.5) mg/kg bw per day compared to control (n = 12.8) and 30 (n = 12.5) mg/kg bw per day. These changes were associated with a reduction in mean fetal body weight in males at 100 (−5 g) and 300 (−12 g) mg/kg bw per day and in mean fetal body weight in females at 30 (−1 g), 100 (−6 g) and 300 (−13 g) mg/kg bw per day, compared to control.

No treatment-related macroscopic changes were identified at any dose. At 300 mg/kg bw per dose, the mean postimplantation loss was high (35.3%) compared to the control group (15.6%) and a statistically significantly higher mean number of live fetuses per female were observed. At 300 mg/kg bw per day, there was a higher mean percentage of resorptions (33.1%) compared to control group (13.5%), associated with a higher percentage of mean postimplantation loss which resulted in a lower mean number of live fetuses.

Sex ratio of the fetuses was not affected. A lower statistically significant mean fetal body weight seen in both sexes at 300 mg/kg bw per day was considered a treatment-related effect. No variations were seen at external examination of fetuses in control and 30 mg/kg bw per day groups. One dam at mid dose and one at high dose each had a fetus with a variation (malrotated limb); two dams at high dose each had a malformed fetus (exencephaly or cleft palate/mandibular micrognathia). In general, there were no variations at soft tissue examination of fetuses in any treatment group. Three dams at 300 mg/kg bw per day had a malformed fetus (cleft palate or small cerebrum). There was a significant increase in dose-related skeletal variations, primarily delayed ossification, in fetuses at all doses. However, delayed ossification at 30 and 100 mg/kg bw per day occurred in the absence of a corresponding reduction in mean fetal body weight. When the mean fetal (F) and litter (L) incidences in delayed ossification at 30 mg/kg bw per day were compared with historical control data, only the parietal bone and the proximal phalanx were just outside the range (litter to fetal incidence, 9.5 : 2.5 vs 8.3 : 1.3 in historical controls). This small difference in incidence did not achieve statistical significance. At 100 mg/kg bw per day, there were statistically significant increases in the incidence of delayed ossification of the skull (supraoccipital, parietal) bones, sternebrae (1st to 4th) and phalanxes (distal and proximal). At 300 mg/kg bw per day, there were marked increases in incidences of delayed ossification (skull, vertebrae, sternebrae and metacarpal bones, phalanxes) compared with both concurrent and historical controls. The malformations occurred at the low dose (4.8% split palate, 4.8% fused sternebrae compared to 0% in the control group), mid dose (5.9% absent lumbar vertebra, 4.8% fused sternebrae compared to 0% in the control group) and high dose (skull, axial skeleton and extremities).

The NOAEL for maternal toxicity was 30 mg/kg bw per day based on clinical signs (round back, piloerection, pale extremities, emaciated appearance and/or hyperactivity/hypoactivity) at 100 mg/kg bw per day.

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. No evidence of teratogenicity was observed (Spezia, 2015a,b).

Rats

In a GLP-compliant developmental toxicity study conducted in accordance with OECD TG 414, Bentz (2015b) administered halquinol BP 80 (26% 5-CL : 72% 5,7-DCL) in 0.5% w/v methylcellulose to time-mated Sprague Dawley rats (24/group) by gavage at doses of 0, 100, 300 or 1000 mg/kg bw per day during gestation days 6–20. Dose selection was based on results from an earlier dose range–finding study (Bentz, 2014a). Halquinol BP 80 concentrations analysed on the first and last day of treatment were within an acceptable range of variation (−8.8% to +2.2%) compared to nominal values (±15% of nominal concentrations). A constant daily dose volume of 5 mL/kg bw was used.

Animals were examined daily for mortality and clinical signs. Body weights were recorded on gestation days 2, 4, 6, 9, 12, 15, 19 and 21 and prior to scheduled or premature kill. Feed consumption was recorded on gestation days 2–4, 4–6, 6–9, 9–12, 12–15, 15–18 and 18–21. On gestation day 21, females were killed and macroscopically and microscopically examined. Uteruses were removed by caesarean section. The number of corpora lutea, implantations, early and late resorptions and live and dead fetuses were recorded. Implantations were classified as live, dead (non-live fetus with discernible digits), a late resorption (dead embryo or fetus with external degenerative changes) or an early resorption (evidence of implant without recognizable embryo). The fetuses were weighed, sexed (visual assessment of the anogenital distance and internal examination of sexual organs) and examined for external, soft tissue and skeletal cartilage abnormalities.

Ptyalism (excess salivation) was observed in 16/19 females at 1000 mg/kg bw per day. At 1000 mg/kg bw per day, one female was euthanized in poor condition. Piloerection was recorded in one surviving pregnant female at 1000 mg/kg bw per day euthanized in poor condition (piloerection, round back, emaciated appearance, walking on tip toe, 16% body-weight loss in 2 days) on gestation day 17 and in one non-pregnant female a few days before scheduled kill. These two females were not included in the clinical signs summary as this included only surviving pregnant females. Green faeces were seen for most of the treatment period in all pregnant females at 300 (20/23) and 1000 mg/kg bw per day (19/19); this was considered a test substance–related effect.

There was no effect on body weight; however, there was a statistically significant body-weight change at 1000 mg/kg bw per day from gestation days 18 to 21 that was considered a test substance–related effect. Mean feed consumption was statistically significantly lower at 1000 mg/kg bw per day throughout the entire treatment (−19% compared with control from gestation day 18 to 21; $P < 0.001$). At 300 mg/kg bw per day, mean feed consumption was statistically significantly lower on gestation days 6–9 (−7% compared with control; $P < 0.001$),

but returned to control values thereafter. No effect on feed consumption was seen at 100 mg/kg bw per day.

Halquinol BP 80 had no effect on mean gravid uterus weight. However, at 1000 mg/kg bw per day, mean net body-weight gain was statistically significantly lower than control from gestation day 6; a similar dose-related decrease was observed at 300 and 100 mg/kg bw per day.

The most relevant necropsy finding in 14 out of 19 pregnant high-dose females at term was dilated caecum. Other effects at the same dose were enlarged cervix and serous content in the uterus and irregular colour of kidneys.

No effect of halquinol BP 80 on implantation loss or number of fetuses per female was seen. At scheduled kill on gestation day 21, there were 23/24, 22/24, 23/24 and 19/23 females with live fetuses at 0, 100, 300 and 1000 mg/kg bw per day, respectively. Body weight of the female fetuses showed a statistically significant dose-related decrease at all doses ($P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively), while body weight of the male fetuses was equally affected but only statistically significantly at 300 and 1000 mg/kg bw per day.

No treatment-related variations or malformations were observed at fetal soft tissue examination. An isolated omphalocele and incidences of enlarged ventricular chamber occurred at 100 mg/kg bw per day and, in the same dose group, the right sides of the aortic and pulmonary trunks were affected. These effects were not considered treatment related. In all treated groups, there were higher incidences of ossification delays (cartilages present) correlating with statistically significant lower mean fetal body weight in females at 100 mg/kg bw per day and both sexes at higher doses. These effects included extra 14th ribs (4.3% and 5.3% litter incidence at 300 and 1000 mg/kg bw per day, respectively); short rib(s) (4.3% and 10.5% litter incidence at 300 and 1000 mg/kg bw per day, respectively); unossified metacarpal bone (9.1% and 10.5% litter incidence at 100 and 1000 mg/kg bw per day, respectively); unossified metatarsal bone (fetal incidence at 23/144, 30/163 and 24/132 and litter incidence at 45.5%, 56.5% and 52.6% at 100, 300 and 1000 mg/kg bw per day, respectively); and unossified distal phalanx (17.4%, 45.5%, 47.8% and 21.1% at 0, 100, 300 and 1000 mg/kg bw per day). In addition, there was an increased incidence of absent cartilage of ribs (10.5% litter incidence). These effects were considered treatment related. One fetus at 1000 mg/kg bw per day had skeletal malformations (fused sternebrae, fused ribs and fused cervical vertebrae arches) for which a treatment-related effect could not be excluded.

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 weight at 100 mg/kg bw per day, the lowest dose tested.

No evidence of teratogenicity was observed (Bentz, 2015b).

Rabbits

In a GLP-compliant maximum tolerated dose, oral toxicity study, Bentz (2014a,b) administered halquinol BP 80 (26% 5-CL : 72% 5,7-DCL) to New Zealand White nonpregnant female rabbits (3/group) by gavage at 300 mg/kg bw per day. A constant dosage volume of 3 mL/kg bw was used. Animals were checked daily for mortality and morbidity during the acclimation period and at least twice a day during the treatment period. The plan was to administer the test item for 10 days, followed by a 9-day wash-out period and another 10 days at a different dose level. However, treatment was stopped on day 8 due to the death of two animals. Before dying, these rabbits lost body weight because of reduced feed consumption and produced no faeces. Blood was found in the cages the day before death, but there were no obvious injuries. A third animal showed the same clinical symptoms and was euthanized on day 9. The main thoracic and abdominal organs of all animals were macroscopically examined.

There was no clear cause of death for the two animals that died during the study; it should be noted that organs were already partially autolysed at the time of examination. The euthanized animal had black content in the gall bladder that was considered a treatment-related effect (Bentz, 2014a,b).

The antibiotic-induced disturbance of the rabbit gut flora and their hypersensitivity specifically to high doses of orally applied antibiotics is well documented (Varga, 2014). The results of the study indicate a test item–induced enterotoxaemia in the rabbits. The obvious hypersensitivity of rabbits towards antimicrobials makes them an inappropriate animal model for toxicity testing of halquinol BP 80. Therefore, the sponsor decided to investigate developmental toxicity in mice as a second species.

2.2.6 Special studies

No special studies such as safety studies were submitted by the sponsor. A few brief reports retrieved in the literature search have been included in the dermal, sensitization and human studies sections of this monograph.

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 VICH guidelines (GL36; VICH, 2004), was adopted by the sixty-sixth JECFA (Annex 1, reference *181*).

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 acceptable daily intake (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 measureable activity against intestinal microbiota.

In a GLP-compliant study, Pridmore (2015) 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 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 (CLSI, 2012a,b, 2014). 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 2. 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

Table 2

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

Test item	Summary MIC parameter	Value (mg/mL) for each bacterial group								
		Enterococcus	*Escherichia coli*	*Clostridium*	*Bacteroides*	*Bifidobacterium*	*Lactobacillus*	*Fusobacterium*	Gram-positive anaerobic cocci	Gram-positive non-spore forming 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	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	>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	All >256	All >256	All >256	64 - >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	>256	>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

Geo. mean: geometric mean; MIC: minimum inhibitory concentration; MIC_{50}: minimum concentration required to inhibit the growth of 50% of organisms; MIC_{90}: minimum concentration required to inhibit the growth of 90% of organisms
Source: Pridmore (2015)

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 2) (Pridmore, 2015).

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 (Costa et al., 2017).

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 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 urine over 72 hours (Berggren & Hansson, 1968). Another study in male rats indicated that about 30% of halquinol and metabolites were excreted in urine (Bories & Tulliez, 1972). A study with pigs using ^{14}C-labelled halquinol indicated that halquinol is rapidly excreted, mainly in urine, with recovery of radioactivity in faeces averaging about 36% (Cosgrove et al., 1981). Based on its similarity to the urinary excretion of halquinol analogues by humans, rat urinary excretion of halquinol determined by Bories & Tulliez (1972) 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 to halquinol (Table 2; Pridmore, 2015). Nevertheless, since intestinal bacteria have hydrolytic enzymes such 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* (Cosgrove et al., 1981). In addition, although halquinol has been in widespread use in human and veterinary medicine, no microbiological studies suggest 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:

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

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). The MIC_{calc} is derived as follows:

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

  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 by Pritchard et al., 2014) 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, and 25% in humans treated with structurally related analogues, according to Berggren & Hansson, 1968), 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:

$$\text{ADI} = \frac{24\ \mu\text{g/mL} \times 500\ \text{mL}}{0.70 \times 60\ \text{kg bw}} = 0.285\ \text{mg/kg bw per daybw}$$

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) (see Table 2).

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

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

$$= \frac{24\ \mu\text{g/mL} \times 3 \times 500\ \text{mL}}{0.70 \times 60\ \text{kg bw}} = 0.857\ \text{mg/kg bw}$$

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

2.4 Observations in humans

Oral formulations of quixalin (trade name Endiaron) have been used in humans for the control of infectious diarrhoea, disorders of the intestinal microflora (e.g.

after antibiotic treatment), giardiasis and inflammatory bowel disease amoebiasis. Halquinol has been used for a long time in the treatment of human amoebic and bacillary dysenteries (Heseltine & Freeman, 1959; Heseltine & Campbell, 1960; Satoskar, Bhandarkar & Rege, 2009). A 1973 clinical trial to determine the efficacy of quixalin tablets in children with dysenteriform diarrhoea determined that halquinol (quixalin) was highly effective in 39/45 cases when used at 30–50 mg/kg bw per day (Damayanti, Nasar & Pudjiadi, 1973). Halquinol in conjunction with corticosteroids is used for its antimicrobial activity in topical preparations in the treatment of skin disorders (Alsterholm, Karami & Faergemann, 2010) and incorporated in shampoo (2% w/v) in the treatment of dandruff and seborrhoeic dermatitis of the scalp. A clinical trial of the use of halquinol to treat impetigo suggests that halquinol cream (0.75%) has an effective antibacterial activity (Readett, 1965, 1966).

In 1972, a patient taking quixalin (halquinol) as directed on the label experienced diarrhoea and numbness in her feet, which later spread to her legs, and had difficulty walking. Her vision deteriorated to the point where she became legally blind. A neurologist diagnosed her with peripheral neuropathy and optic atrophy most likely associated with treatment with halquinol (Hansson & Herxheimer, 1981).

Halquinol orally administered to humans at 70 mg/kg bw for 10 days did not result in any signs of toxicity (Freeman & Heseltine, 1963).

Sensitization was reported in a worker who helped add Quixalud to feeds for 2 weeks. A patch test with 1% Quixalud (60% halquinol, 40% chalk) gave marked positive results (Burrows, 1975).

3. Comments

The Committee considered data on pharmacokinetics, short-term toxicity and long-term toxicity, reproductive and developmental toxicity, genotoxicity and microbiological safety. No carcinogenicity studies were submitted. Most of the submitted studies complied with GLP. A literature search identified 20 papers with information additional to what was submitted.

3.1 Biochemical data

None of the submitted studies considered the pharmacokinetics of halquinol. However, plasma levels of 5,7-DCL and 5-CL were measured in the 4- and 13-week rat (Bentz, 2015a; Chevalier, 2015) and mini-pig (Chevalier, 2014a,b) toxicity studies and the 13- and 39-week dog (El Amrani-Callens, 2016a,b,

2017) toxicity studies. Other kinetic information was obtained using read-across from structurally similar compounds such as chloro-7-iodoquinolin8-ol and 5,7-dibromoquinolin-8-ol in rats, guinea-pigs and rabbits (Haskins & Luttermoser, 1953; Hayashi et al., 1976; Kiwada et al., 1977; Sawada et al., 1978; Kotaki, 1983). 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. In vitro incubation of [^{14}C]5,7-DCL with isolated hepatocytes prepared from rats, dogs, mini-pigs, 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-DCL (Novo, 2015a,b).

The predominant conjugate (sulfate or glucuronide) found in urine or bile/faeces differed between 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, whereas most of the remainder (69%) was detected in faeces less than 48 hours after dosing (Bories & Tullierz, 1972). 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 liver, kidneys, muscle or fat (Bories & Tullierz, 1972).

3.2 Toxicological data

Critical studies relevant to the risk assessment are summarized in Table 3.

The acute toxicity of halquinol has been investigated in mice and rats. The oral LD_{50} was 470–850 mg/kg bw in mice and 700 mg/kg bw in rats (Boissier, 1959; Eckert, 1961; Newman, 1963; Hurd, 1964; Adams, 1973). 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 (Sire, 2014a).

In a non-GLP-compliant 28-day oral toxicity study in rats, halquinol was administered by gavage at doses of 0, 150, 450 or 1000 mg/kg bw per day. The NOAEL was 150 mg/kg bw per day based on histopathological changes in liver (focal necrosis, congestion, hydropic degeneration) and kidney (cystic dilation of collecting tubules, necrosis of tubular epithelial lining) associated with a significant increase in clinical parameters (Swetha, 2007).

Table 3
Studies relevant to risk assessment

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				
Thirteen-week toxicity (gavage)	0, 50, 150, 450	Histopathological lesions in the kidneys	50	150
One-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	–
Developmental study (gavage)	0, 100, 300, 1 000	Maternal toxicity: Clinical signs	300	1 000
		Embryo and fetal toxicity: Lower mean fetal body weights correlating with delayed bone ossification	–	100
Dog				
Thirteen-week toxicity study (gelatin capsule)	0, 3, 10, 60, 150	Body weight loss	30 [a]	60
Thirty-nine-week toxicity study (gelatin capsule)	0, 30, 60, 90	Body weight loss	30 [a]	60

[a] Overall NOAEL

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 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, histopathological changes in kidney and lymph nodes (Chevalier, 2015).

In a 13-week oral toxicity study in rats with a 4-week recovery period, halquinol was administered by gavage at 0, 50, 150 or 450 mg/kg bw per day. The NOAEL was 50 mg/kg bw per day based on increase in kidney weights (absolute and relative) associated with histopathological changes (Bentz, 2015a).

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 0, 30, 60 or 90 mg/kg bw per day for 39 weeks. An overall NOAEL of 30 mg/kg bw per day was established based on lower terminal body weights at 60 mg/kg bw per day in males (El Amrani-Callens, 2016a,b, 2017).

In a 1-year chronic toxicity study in rats, halquinol was administered by oral gavage at 0, 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 (Chevalier, 2017).

Halquinol and its conjugates were tested in a range of in vitro tests including bacterial reverse cell mutation tests (Nagao, 1977; Frieauff, 2013a,b,c,d), mammalian cell mutation tests using L5178Y $tk^{+/-}$ mouse lymphoma cells (Sire, 2014a) and chromosomal aberration tests in cultured lymphocytes (Sire, 2014b). Halquinol tested positive in mouse lymphoma and chromosome aberration tests in vitro (Sire, 2014b). Two adequately conducted in vivo studies examining the potential of halquinol to induce chromosome damage in rat bone marrow cells were negative (Ciliutti, 2014; Sire, 2014a). The Committee concluded that the positive clastogenicity observed in vitro was not confirmed in vivo. However, the positive result in the mammalian mutagenicity study in vitro was not addressed by the in vivo studies (micronucleus induction and chromosome aberration), both of which were performed using bone marrow, because they did not assess the induction of a mutation. Moreover, structure–activity relationship (SAR) analysis indicated the presence of structural alerts for both mutagenicity and clastogenicity for all three components of halquinol.

In the absence of a carcinogenicity study or an in vivo gene mutation study, the Committee was unable to reach a definitive conclusion with respect to the genotoxic potential of halquinol.

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 F_0 and F_1 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 (Spezia, 2016).

In a developmental toxicity study in mice, halquinol was administered by oral gavage on gestation days 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 and/or hyperactivity/hypoactivity) at 100 mg/kg bw per day. 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. No evidence of teratogenicity was observed (Spezia, 2015a,b).

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 (Bentz, 2015b).

The Committee concluded that halquinol was not teratogenic in mice and rats.

3.3 Observations in humans

No reliable information on toxicological effects of halquinol in humans was available.

3.4 Microbiological data

A decision-tree approach adopted by the sixty-sixth meeting of the Committee (Annex 1, reference *181*) that complies with VICH GL36 (VICH, 2004/18, 19) was used by the Committee to determine the need for and to establish, if necessary, an mADI for halquinol. Studies of microbiological activity of halquinol against bacterial strains representative of the human colonic microbiota were evaluated. Halquinol was active against *E. coli* (MIC_{50} = 16 µg/mL), *Bifidobacterium* (MIC_{50} = 256 µg/mL), *Clostridium* (MIC_{50} = 32 µg/mL), *Bacteroides* (MIC_{50} = 32 µg/mL), *Lactobacillus* (MIC_{50} = 64 µg/mL), *Fusobacterium* (MIC_{50} = 32 µg/mL), *Eubacterium* (MIC_{50} = 32 µg/mL) and *Peptostreptococcus* (MIC_{50} = 32 µg/mL).

Halquinol residues would be microbiologically active in the human gastrointestinal tract. However, halquinol is extensively biotransformed to conjugates with considerably-reduced-to-no activity before reaching the colon in humans. The MIC_{50} values for the sulfate and glucuronide conjugates of 5-CL and 5,7-DCL were greater than 256 µg/mL. However, because intestinal bacteria have hydrolytic enzymes such as β-glucuronidase and arylsulfatase, there is potential for the halquinol metabolites in the gastrointestinal tract to be deconjugated back to the microbiologically active halquinol.

Halquinol does not appear to induce or select for the development of bacterial resistance. Results from a study in which halquinol was fed to pigs over a 6-week period showed no induction of resistance to *E. coli* (Cosgrove et al., 1981). Despite its long use in human and veterinary medicine, resistance development to halquinol has not been reported. Because resistance development is unlikely, the only potential adverse effect on human intestinal microbiota is disruption of the colonization barrier. Consequently, an mADI for halquinol residues was derived.

The formula for deriving the mADI is as follows:

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

where:

- MIC_{calc}: In accordance with Appendix C of VICH GL36 (VICH, 2004, 2012), 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 strains needed to determine the MIC_{calc} for halquinol were chosen according to these guidelines, which state that data on an intrinsically resistant bacterial genus should not be included in the calculation. Based on the MIC_{50} values (see above), the MIC_{calc} was 24.0395 µg/mL.
- *Mass of colon content:* The 500 mL value is based on the colon volume measured in humans (Pritchard et al., 2004).
- *Fraction of oral dose available to the microorganisms:* It is recommended that the fraction of an oral dose available for colonic microorganisms be based on in vivo measurements for the drug administered orally. Alternatively, if sufficient data are available, the fraction of the dose available for colonic microorganisms can be calculated as 1 minus the fraction (of an oral dose) excreted in urine. Human data are encouraged, but in their absence, 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 that of the parent compound. The fraction may be lowered if the sponsor provides quantitative in vitro or in vivo data to show that the drug is inactivated during transit through the intestine.
- Halquinol is rapidly absorbed and is excreted in urine or bile, primarily conjugated with sulfate or glucuronide. The lowest halquinol urinary recovery data from rat studies was 30% (Bories & Tulliez, 1972). Similar recoveries were observed in the urine of humans treated with structurally related analogues. Therefore, based on similar rates of excretion in rats and humans, the fraction of a halquinol oral dose available in the colon would be 1–0.30 = 0.70.
- The body weight of an adult human is assumed to be 60 kg.

The upper bound of the mADI for halquinol was calculated as follows:

$$\text{Upper bound of ADI} = \frac{24\ \mu g/mL \times 500\ mL}{0.70 \times 60\ kg\ bw} = 0.285\ mg/kg\ bw$$

The microbiological ARfD for halquinol was determined using the following formula:

$$\text{Microbiological ARfD} = \frac{(\text{MIC}_{\text{calc}} \text{ or NOAEC}) \times \text{Correction factors} \times \text{Colon volume}}{\text{Fraction of oral dose available to microorganisms} \times 60 \text{ kg}}$$

$$= \frac{24 \text{ µg/mL} \times 3 \times 500 \text{ mL}}{0.70 \times 60 \text{ kg bw}} = 0.857 \text{ mg/kg bw}$$

4. Evaluation

The Committee concluded that a toxicological ADI cannot be established due to the lack of information required to assess the in vivo mutagenicity and carcinogenicity potential of halquinol.

For the effects on the colonization barrier, an mADI of 0–0.285 mg/kg bw, rounded to 0–0.3 mg/kg bw, was derived from in vitro MIC susceptibility testing data and using the newly adopted colon volume of 500 mL. A microbiological ARfD of 0.857 mg/kg bw, rounded to 0.9 mg/kg bw, was established based on the effects of halquinol on the intestinal microbiota and using the newly adopted colon volume of 500 mL.

It was not possible to establish an ADI for halquinol in the absence of a toxicological ADI.

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Lufenuron

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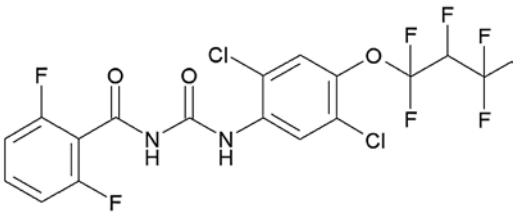
1. Explanation

Lufenuron (Fig. 1) is the International Organization for Standardization–approved common name for (*RS*)-1-[2,5-dichloro-4-(1,1,2,3,3,3-hexafluoropropoxy) phenyl]-3-(2,6-difluorobenzoyl)urea (International Union of Pure and Applied Chemistry), for which the Chemical Abstracts Service number is 103055-07-8.

Lufenuron is an insecticide initially registered for use on a wide range of crops for the control of the larvae of many insect pests. Lufenuron inhibits chitin synthesis, probably through enzymatic interference, and prevents the larvae from moulting.

Fig. 1
Structure of lufenuron



Lufenuron has not been previously evaluated by the Joint FAO/WHO Expert Committee for Food Additives (JECFA) but was reviewed at the present Meeting at the request of the Twenty-third Session of the Codex Committee on Residues of Veterinary Drugs in Foods (FAO/WHO, 2016). The Committee was asked to establish an acceptable daily intake (ADI) and recommend maximum residue limits for lufenuron in fin fish (salmon/trout) muscle and skin in natural proportions.

The Committee noted that lufenuron had been previously evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR; FAO/WHO, 2015). At that meeting, JMPR established an ADI of 0–0.02 mg/kg body weight (bw) for lufenuron, based on the no-observed-adverse-effect level (NOAEL) of 1.93 mg/kg bw per day for tonic–clonic seizures and findings in lungs, gastrointestinal tract, liver and urinary tract in the 2-year dietary study in rats (FAO/WHO, 2015). JMPR also concluded that it was not necessary to establish an acute reference dose for lufenuron 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.

Because the review of lufenuron by JMPR was so recent (in 2015), a literature search on lufenuron was not undertaken. The JECFA evaluation is essentially identical to the JMPR evaluation with minor editorial changes to accommodate formatting differences. In addition, a few unpublished studies previously not available to JMPR have been included in this review. These unpublished studies relate to the acute median lethal dose (LD_{50}) and genotoxicity of two lufenuron metabolites, CGA 224443 and CGA 149772. Metabolite CGA 224443 is a minor component (<1%) of lufenuron metabolism in rats, whereas metabolite CGA 149772 is reported to be found in soil. A short-term 28-day dietary study for CGA 224443 in rats was also submitted. The only additional study for lufenuron was a pharmacokinetic and mass balance study in dogs. All critical studies contained statements of compliance with good laboratory practice

(GLP). Overall, the present Committee considered that the database was adequate to assess the risk of lufenuron.

2. Biological data

2.1 Biochemical aspects

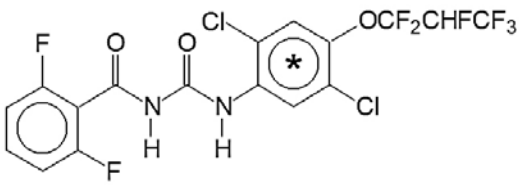
2.1.1 Absorption, distribution and excretion

(a) Oral administration

Rats

To investigate the absorption, distribution and excretion of lufenuron, a single oral dose of [^{14}C]lufenuron (batch no. ILA-178 7A; >98% radiochemical purity; see Fig. 2 for position of the radiolabel) was administered by gavage to four groups of four male rats (Alpk:AP$_f$SD, Wistar-derived) at a dose of 0.1, 1.0, 10 or 100 mg/kg bw, or a single intravenous dose of [^{14}C]lufenuron was administered to two groups of four male rats at a dose of 0.1 or 10 mg/kg bw. The dose vehicle was 7:3 (volume per volume [v/v]) polyethylene glycol (PEG) 400/ethanol. Urine and faeces were collected daily for 7 days following dosing. Subsequently, faeces were collected daily on days 8–21 following dosing, and urine was collected on days 10–11, 14–15 and 19–21. Serial blood samples were collected at 1, 2, 4, 8, 12, 24, 48, 72, 96, 120, 144, 168, 288 and 384 hours after dosing and by cardiac puncture at termination. Twenty-one days after dosing, each rat was terminated.

Fig. 2
Position of radiolabel on [^{14}C]lufenuron



* = ^{14}C position

The study was not performed according to GLP, but it met all criteria specified in Organisation for Economic Co-operation and Development (OECD) Test Guideline 417.

Table 1
Comparison of kinetics following single oral or intravenous administration of lufenuron

Route	Dose (mg/kg bw)	Radioactivity (% of dose at 21 days after dosing)				
		Urine	Faeces	Tissue and residual carcass	Total recovery	$AUC_{0-120\ h}$ (h•µg eq./g)
Oral	0.1	0.64	80.59	21.00	102.93	0.40
	1.0	0.51	72.93	23.55	97.23	4.37
	10	0.57	80.37	14.83	96.05	41.25
	100	0.17	91.87	5.17	97.43	83.88
Intravenous	0.1	0.64	66.50	32.03	99.77	0.56
	10	0.82	62.23	34.73	98.11	60.69

$AUC_{0-120\ h}$: area under the blood concentration–time curve over 120 hours; bw: body weight; eq.: equivalents
Source: Booth (2004)

Over the 21-day duration of the study, the mean total proportion of administered radioactivity excreted in urine following oral dosing at 0.1, 1.0, 10 or 100 mg/kg bw was 0.6%, 0.5%, 0.6% and 0.2%, respectively, whereas faecal excretion accounted for 80.6%, 72.9%, 80.4% and 91.9%, respectively. The mean total percentage of dose excreted in urine following an intravenous dose of 0.1 or 10 mg/kg bw was 0.6% and 0.8%, respectively, whereas faecal excretion accounted for 66.5% and 62.2%, respectively. A characteristic feature of faecal excretion was the slow, sustained period of elimination. Faecal excretion was still measurable 21 days following administration of a single oral or intravenous dose, irrespective of dose level (Table 1).

The area under the blood concentration–time curves over 120 hours ($AUC_{0-120\ h}$) after dosing, for both dose routes, clearly show that for dose levels between 0.1 and 10 mg/kg bw, systemic exposure is directly proportional to the administered dose. The systemic bioavailability of lufenuron can be directly determined by the classical method of calculating the ratio of AUC between an oral and an intravenous dose. This gives a value of approximately 70% for the 0.1 and 10 mg/kg bw dose levels. The amount of radioactivity in urine following oral and intravenous dosing can also be treated similarly to obtain an estimate of absorption. For the 10 mg/kg bw dose level, the urinary data indicate an absorption value of 70%; for the 0.1 mg/kg bw dose level, the absorption is estimated at 100%.

The blood $AUC_{0-120\ h}$ increased with dose over the range 0.1–100 mg/kg bw. At the highest oral dose level, 100 mg/kg bw, evidence of saturation was observed, and the AUC was no longer proportional to the administered dose. Saturation was more evident in the relationship between blood $AUC_{0-\infty}$ and dose. The time to reach the maximum concentration (T_{max}) was 8 hours after oral administration and 2 hours after intravenous administration (Table 2).

Table 2
Blood kinetics data following a single oral or intravenous dose of [^{14}C]lufenuron

Nominal dose, route	Mean achieved dose (mg/kg bw)	$AUC_{0–120\ h}$ (h•µg/g)	$AUC_{0–\infty}$ (h•µg/g)	T_{max} (h)	C_{max} (µg/g)
0.1 mg/kg bw, oral gavage	0.10	0.40	0.49	8	0.008
1.0 mg/kg bw, oral gavage	1.08	4.37	10.73	8	0.097
10 mg/kg bw, oral gavage	10.52	41.25	90.44	8	0.89
100 mg/kg bw, oral gavage	101.85	83.88	216.36	8	1.341
0.1 mg/kg bw, intravenous	0.11	0.56	0.94	2	0.017
10 mg/kg bw, intravenous	10.35	60.69	153.76	2	1.907

$AUC_{0–120\ h}$: area under the blood concentration–time curve over 120 hours; $AUC_{0–\infty}$: area under the blood concentration–time curve from time 0 to infinity; bw: body weight; C_{max}: maximum concentration; T_{max}: time to reach maximum concentration
Source: Booth (2004)

The mean terminal half-life of faecal excretion following oral dosing was 256 hours, and following intravenous dosing, 232 hours. At termination of the study, the mean total proportion of administered radioactivity present in tissues and carcass following oral dosing at 0.1, 1.0, 10 or 100 mg/kg bw was 21.0%, 23.6%, 14.8% and 5.2%, respectively. The mean total proportion of dose present in tissues following an intravenous dose of 0.1 or 10 mg/kg bw was 32.0% and 34.7%, respectively. Radioactivity was located mainly in the fat and, to a much lesser extent, in residual carcass, followed by liver and kidneys. The total mean percentage recoveries of administered radioactivity, including excreta, tissues and residual carcasses, following oral dosing at 0.1, 1.0, 10 or 100 mg/kg bw were 102.9%, 97.2%, 96.0% and 97.4%, respectively. The total mean percentage recoveries of administered radioactivity, including excreta, tissues and residual carcasses, following an intravenous dose of 0.1 or 10 mg/kg bw were 99.8% and 98.1%, respectively.

From the AUC values determined for blood, the dose–response curve does not appear to be linear at the high dose level following oral gavage dosing (Booth, 2004).

To investigate the absorption, distribution and excretion of lufenuron, [^{14}C]lufenuron (batch no. Le-78.3A; approximately 99% radiochemical purity) was administered by gavage to groups of male and female rats (Tif: RAIf (SPF)) at a single dose of either 0.5 or 100 mg/kg bw following the study design indicated in Table 3.

Irrespective of dose level or sex, less than 1% of an oral dose of [^{14}C] lufenuron was excreted in urine, and less than 0.01% of the administered radioactivity was detected in exhaled volatile and carbon dioxide traps. The extent of absorption of a 0.5 mg/kg bw dose was 44%, based upon radioactivity present in urine and residual carcass and tissues at 7 days after administration. However, the extent of absorption and faecal excretion was dose dependent. Despite the

Table 3
Terminal toxicokinetic studies in the rat – experimental scheme

Group	Number and sex	Route and dose level of [^{14}C]lufenuron	Sample collection times
B	5 males, 5 females	Single oral dose of 0.5 mg/kg bw 199 kBq/animal	Urine: 0–8, 8–24, 24–48, 48–72, 72–96, 96–120, 120–144 and 144–168 h Faeces: 0–8, 8–24, 24–48, 48–72, 72–96, 96–120, 120–144 and 144–168 h Volatiles: 0–8 and 8–24 h Expired air: 0–8 and 8–24 h Tissues: 7 days after administration of the ^{14}C-labelled material, tissues and organs were taken for analysis
C	5 males, 5 females	Single oral dose of 0.5 mg/kg bw [^{14}C] lufenuron preceded by 14 consecutive daily oral doses of 0.5 mg unlabelled lufenuron/kg bw 239 kBq/animal	Collection of urine, faeces, volatiles, expired air and tissues as in Group B
D	5 males, 5 females	Single oral dose of 100 mg/kg bw 7 785 kBq/animal (diluted with batch no. P1 to a specific activity of 385 kBq/mg)	Collection of urine, faeces, volatiles, expired air and tissues as in Group B
E1	3 males	Single oral dose of 0.5 mg/kg bw 196 kBq/animal	Blood: 1, 2, 4, 8, 12, 24, 32, 48 and 56 h after administration (determination of T_{max} and T_{max}/2 of this dose)
E2	3 males	Single oral dose of 100 mg/kg bw 1 423 kBq/animal (diluted with batch no. AMS 266/101 to a specific activity of 72 kBq/mg)	Blood: 1, 2, 4, 8, 12, 24, 32, 48, 56 and 72 h after administration (determination of T_{max} and T_{max}/2 of this dose)
F1 and F2	10 males	Single oral dose of 0.5 mg/kg bw 191 kBq/animal	Tissues: 8 h (T_{max}), 45 h (T_{max}/2), 5 days and 12 days after administration, selected tissues and organs were taken for analysis
F3 and F4	10 males	Single oral dose of 100 mg/kg bw 1 378 kBq/animal (diluted with batch no. AMS 266/101 to a specific activity of 70 kBq/mg)	Tissues: 8 h (T_{max}), 60 h (T_{max}/2), 5 days and 12 days after administration, selected tissues and organs were taken for analysis
G	5 males, bile duct cannulated under anaesthesia	Single oral dose of 0.5 mg/kg bw 237 kBq/animal	Urine: 0–24 and 24–48 h Faeces: 0–24 and 24–48 h Bile: 0–0.5, 0.5–1, 1–2, 2–4, 4–8, 8–18, 18–24, 24–42 and 42–48 h

bw: body weight; T_{max}: time to reach maximum concentration; $T_{max/2}$: time to decline to half the maximum concentration
Source: Bissig (1990)

similarities in faecal excretion of a 0.5 mg/kg bw dose by bile duct–cannulated and non-cannulated male rats and the low biliary elimination over 48 hours after dosing, the slow rate of faecal excretion by non-cannulated rats suggests that some faecal radioactivity was of biliary origin. The extent of absorption of a 100 mg/kg bw dose was much lower, accounting for at least 9% of the dose, based mainly on radioactivity present in tissues at the termination of the experiment. Even though some of the systemic dose was likely to have been eliminated in bile, the much

Table 4
Excretion of lufenuron by rats following an oral [^{14}C]lufenuron dose of 0.5 or 100 mg/kg bw

		Excretion (% of administered radioactivity)						
		B (Single dose 0.5 mg/kg bw)		C (Repeated dose 0.5 mg/kg bw per day)		D (Single dose 100 mg/kg bw)		G (Bile duct cannulated, single dose 0.5 mg/kg bw)
		Males	Females	Males	Females	Males	Females	Males
Bile	0–8 h	–	–	–	–	–	–	0.33
	8–24 h	–	–	–	–	–	–	0.54
	24–48 h	–	–	–	–	–	–	0.82
	Subtotal	–	–	–	–	–	–	1.70
Urine	0–24 h	0.27	0.29	0.19	0.31	0.10	0.14	0.09
	24–48 h	–	–	–	–	–	–	0.08
	24–96 h	0.35	0.27	0.25	0.28	0.10	0.10	–
	96–168 h	0.20	0.16	0.16	0.17	0.05	0.04	–
	Subtotal	0.82	0.72	0.60	0.75	0.26	0.28	0.17
Faeces	0–24 h	25.97	23.71	38.83	23.27	66.88	73.20	8.57
	24–48 h	–	–	–	–	–	–	43.03
	24–96 h	17.71	15.12	11.17	12.93	13.44	8.75	–
	96–168 h	8.26	8.92	5.31	7.84	2.10	1.32	–
	Subtotal	51.95	47.74	55.31	44.04	82.42	83.26	51.60
Expired air	0–24 h	Not measured	Not measured	Not measured	Not measured	<0.01	<0.01	Not measured
Total excretion		52.77	48.46	55.91	44.79	82.68	83.54	53.47
Cage wash		0.07	0.07	0.05	0.06	0.08	0.14	–
Tissue residues		5.41	4.95	5.87	8.36	1.96	1.50	–
Carcass		38.09	43.80	37.09	44.42	9.65	7.42	–
Total recovery	0–168 h	96.34	97.27	98.92	97.64	94.37	92.59	53.47

bw: body weight
Source: Bissig (1990)

lower proportion of dose retained in tissue and the very low urinary excretion indicate that at the high dose level, the capacity to absorb the test material was reduced (Table 4).

The urinary excretion and tissue residue data showed that at least 44% and 9% of the orally administered lufenuron were absorbed from the intestinal tract into the systemic circulation by the low-dose rats (groups B and C) and the high-dose rats (group D), respectively.

Seven days after a single oral dose of 0.5 mg/kg bw, significant residues were present in most tissues in both sexes, predominantly in fat (1.91 parts per million [ppm] in males and 2.40 ppm in females). Residues above 0.1 ppm were also measured in ovaries (0.44 ppm), uterus (0.23 ppm), thyroid (0.22 ppm

in males and 0.16 ppm in females), liver (0.13 ppm in males and 0.15 ppm in females), lungs (0.11 ppm in females) and kidneys (0.11 ppm in females). As a consequence of the slightly faster excretion by males, the residues in virtually all tissues in males in groups B and C were slightly lower than those in females, with the single exception of the thyroid in group B. As approximately 43–49% of a single 0.5 mg/kg bw dose of [^{14}C]lufenuron was present in tissues, predominantly fat, after 7 days and as similar proportions of a 0.5 mg/kg bw dose of radiolabelled lufeuron were present in tissues of rats pretreated with 14 daily doses of unlabelled lufenuron, substantial proportions of each unlabelled dose are also likely to be present in tissues. In contrast, 9–12% of a 100 mg/kg bw dose remained in tissues 7 days after dosing, reflecting the lower extent of absorption. Accordingly, at this dose level, tissue residues were only 40–60 times and 30–40 times higher in males and females, respectively. The only exception to this observation was thyroid in females, which contained approximately 100 times higher residue levels. As for the low dose level, the highest residue levels were present in the fat.

The blood kinetics of rats in groups E1 and E2 (single oral doses of 0.5 and 100 mg/kg bw, respectively) showed fairly rapid absorption of lufenuron from the intestinal tract into the systemic circulation. At both dose levels, the maximum blood levels (C_{max}) were reached about 8 hours after dosing. The blood concentrations declined to half of the maximum value ($C_{max/2}$) about 45 and 60 hours ($T_{max/2}$) after dosing at the low and high dose levels, respectively.

At the low dose level, the rate of tissue depletion followed first-order kinetics (45 hours – 12 days; with a coefficient of correlation $r^2 > 0.97$ for most tissues). Tissue concentrations for both low and high doses demonstrated similar depletion kinetics for all tissues. Half-lives of elimination, expressed over two time intervals, showed an initial faster rate followed by a slower terminal rate. Irrespective of dose level or tissue concentration, the initial rates of tissue elimination were similar across the range of tissues analysed, ranging from 2 to 6 days. At the low dose level, the terminal half-lives ranged between 5 and 13 days; the corresponding values at the high dose level ranged from 10 to 37 days (Bissig, 1990).

To investigate the absorption and depletion kinetics of lufenuron, a single oral dose of [^{14}C]lufenuron (batch no. Le-78.3A-1; 97% radiochemical purity) was administered by gavage to two groups of three male and three female rats (Tif: RAIf (SPF)) at a dose of 0.5 or 100 mg/kg bw.

In both sexes, the peak blood concentration of radioactivity was reached 8 and 12 hours after administration of the low and high dose levels, respectively. The ^{14}C concentration in blood declined to half of these values after 56 and 42 hours ($T_{max/2}$) at the low dose level and after 86 and 68 hours at the high dose level in males and females, respectively. Assuming first-order kinetics, a monophasic decline of radioactivity in blood was observed at both the low and high dose

Table 5
Blood kinetic data following a single oral [^{14}C]lufenuron dose of 0.5 or 100 mg/kg bw

	0.5 mg/kg bw		100 mg/kg bw	
	Males	Females	Males	Females
C_{max} (ppm)	0.033	0.035	1.331	1.176
T_{max} (h)	8	8	12	12
Calculated $t_{½}$ (h)	84	97	119	111
$AUC_{0–168\,h}$ (h•µg/g)	2.5	2.5	116.3	105.8

$AUC_{0–168\,h}$: area under the concentration–time curve over 168 hours; bw: body weight; C_{max}: maximum concentration; ppm: parts per million; $t_{½}$: half-life; T_{max}: time to reach maximum concentration
Source: Müller (1995)

levels. When applying linear regression, the calculated half-lives of depuration were 84 and 97 hours at the low dose level and 119 and 111 hours at the high dose level for male and female rats, respectively (Table 5).

The shapes of the areas under the concentration–time curves ($AUC_{0–168\,h}$) were essentially identical for both sexes at both dose levels, accounting at the low dose level for 2.5 h·µg/g. At the high dose level, the respective values were 116 and 106 h·µg/g for male and female rats, respectively (Table 5). When comparing the AUCs at the low and high dose levels, a 46-fold increase in males and a 42-fold increase in females were observed, indicating that a smaller proportion of the dose was bioavailable at the higher dose level. The AUC values were virtually the same for both sexes, demonstrating similar absorption of lufenuron by both males and females (Müller, 1995).

To investigate the absorption, distribution and excretion of lufenuron after multiple oral administrations, [^{14}C]lufenuron (batch no. ILA-178.3; 97.0% radiochemical purity) was administered by gavage to four groups of four male rats (HanBrl: WIST (SPF)). The dosing was performed over 14 consecutive days at a dose of 0.5 mg/kg bw. One group of four males was killed after 1 day of dosing, another group of four males after 7 days of dosing, and the remaining two groups after 14 days of dosing.

Approximately 1% of the dose was excreted in urine, and 58% in faeces. Approximately 38% of the cumulative dose remained in tissues and the residual carcass at the conclusion of this study. The total recovery of administered radioactivity was greater than 97%. The unexcreted dose was accumulated in tissues, and the rate of faecal excretion suggested that such tissue residues would be eliminated slowly and predominantly in faeces. About 80% of the administered dose was found in tissues and residual carcass 1 day after a single oral dose, which indicates that absorption of a 0.5 mg/kg bw single oral dose was approximately 80%.

With the single exception of testes, all tissue concentrations of radioactivity increased to a maximum 1 day after the final dose. The results suggest that most tissue concentrations would plateau within 2–3 weeks of similar repeated dosing. The highest residues were present in fat (29 ppm lufenuron equivalents). Much lower residue levels were present in adrenals (4.2 ppm), pancreas (3.2 ppm), thyroid (3.0 ppm), liver (2.1 ppm), kidneys (1.3 ppm), heart (1.2 ppm), lungs (1.1 ppm) and thymus (1.1 ppm). All other tissues attained maximum levels below 1 ppm lufenuron equivalent. The calculated half-life ($t_{½}$) for the depuration of tissue ^{14}C residues, assuming a monophasic first-order kinetics, typically ranged from 7 to 12 days. A faster depletion was observed for thyroid ($t_{½}$ = 4 days). Elimination half-lives of residues from testes, lungs and fat ranged from 14 to 16 days, although testes showed lower residues after 14 days than after 7 days during the accumulation phase (Table 6).

Seven days after the last of 14 consecutive daily doses, tissue residues represented approximately 38% of the total administered radioactivity. There was a similar distribution pattern between these profiles and those seen 7 days after a similar single oral dose, and there was an approximate 10-fold difference in the comparative tissue concentrations (Table 6).

To conclude, the rates and routes of excretion and the tissue distribution did not change upon multiple dosing compared with single dosing. The tissue residues increased with ongoing dosing, not reaching a plateau within the dosing period. Indication is given that residue levels in most of the tissues will reach a plateau within a dosing period of 3 weeks (Hassler, 2003).

To investigate the absorption, excretion, tissue distribution and pharmacokinetics of lufenuron after multiple oral administrations, [^{14}C]lufenuron (batch no. ISL-158.1; 97.8% radiochemical purity) was administered by gavage to groups of male and female rats (Sprague Dawley Crj:CD(SD)) for 1, 7 or 14 days, respectively. The dosing was performed by 14 consecutive daily doses at a dose of 0.5 mg/kg bw. This study was not compliant with GLP, but it was conducted in Japan to standards apparently equivalent to GLP.

Concentrations of radioactivity in blood measured 8 hours after each dose showed a progressive increase during and following the 14-day repeated-dose experiment, corresponding to the peak blood concentration following a single similar dose. After an initial rapid increase, blood concentrations rose gradually to maximum values of 0.184 and 0.178 µg equivalents of lufenuron per millilitre after the final dose. Concentrations then declined gradually, with estimated elimination half-lives of 208 and 323 hours in males and females, respectively. There was considered to be no marked sex difference in accumulation or depletion profiles.

Excretion by 168 hours after the final dose in male and female rats administered 14 daily oral [^{14}C]lufenuron doses of 0.5 mg/kg bw was 58% of the

Table 6

Tissue residues of radioactivity during and following 14 daily oral [^{14}C]lufenuron doses of 0.5 mg/kg bw

	Tissue residues (µg eq./g tissue)					
	Single dose[a]	Multiple doses				
Organ / matrix	Day 7[b]	Day 1[b]	Day 7[b]	Day 14[b]	Day 20[b,c]	$t_{½}$ (days)
Adrenals	n.a.	0.741 8	2.379 3	4.187 9	2.391 5	7
Blood	0.008 4	0.031 2	0.110 5	0.165 6	0.099 1	8
Bone	0.038 9	0.095 7	0.302 1	0.329 7	0.235 1	12
Brain	0.013 1	0.031 3	0.111 3	0.139 6	0.081 6	8
Fat	1.914 2	3.480 9	21.150 2	29.245 2	22.660 2	16
Heart	0.080 2	0.261 4	0.926 1	1.247 2	0.774 6	9
Kidneys	0.087 9	0.292 3	1.067 7	1.348 1	0.884 5	10
Liver	0.129 2	0.462 0	1.600 5	2.119 2	1.346 0	9
Lungs	0.094 2	0.299 4	0.826 6	1.096 3	0.833 8	15
Muscle	0.040 4	0.156 4	0.576 4	0.636 7	0.395 6	9
Pancreas	n.a.	0.595 7	2.164 5	3.169 6	2.165 1	11
Plasma	0.010 4	0.039 5	0.138 9	0.231 7	0.130 7	7
Spleen	0.046 5	0.160 0	0.547 7	0.724 2	0.512 6	12
Testes	0.026 0	0.082 2	0.367 3	0.279 0	0.207 6	14
Thymus	0.056 0	0.173 4	0.728 4	1.087 0	0.618 5	7
Thyroids	0.220 0	0.413 1	2.441 2	3.023 6	1.097 5	4

bw: body weight; eq.: equivalents; n.a.: not available; ppm: parts per million; $t_{½}$: half-life
[a] From Bissig (1990).
[b] Days after start of dosing.
[c] Seven days after last dose
Source: Hassler (2003)

cumulative dose in males and 64% in females. There was no pronounced sex difference in excretion. Urinary excretion accounted for only about 1% of the cumulative dose in both males and females, whereas faecal excretion accounted for the remainder. The unexcreted dose was accumulated in tissues, and the rate of faecal excretion suggested that such tissue residues would be eliminated slowly and predominantly in faeces.

Concentrations of radioactivity during and following 14 daily oral [^{14}C] lufenuron doses of 0.5 mg/kg bw showed an increase in all tissues until 8 hours after the 14th dose and then a decline. All tissue concentrations exceeded the plasma concentration at all time points, although concentrations in the cerebrum and cerebellum were only slightly higher than those in plasma. The greatest accumulation was observed in fat (28.1 µg eq./g in males and 34.7 µg eq./g in females), with markedly lower peak concentrations in other tissues. Progressively lower peak levels were present in adrenal glands, liver, skin, thyroid, lungs, heart and pituitary. All other tissue concentrations attained peak values of less than

1 µg eq./g. Over the 7 days after the cessation of dosing, all tissue concentrations declined steadily, including fat residues. By this time, the residual carcasses contained 34.6% and 29.5% of the total administered radioactivity in males and females, respectively. There was no marked sex difference in either accumulation or tissue depletion.

Radioluminograms showed very little radioactivity in the brain 8 hours after the first dose. For both sexes, the concentration in the pituitary increased only by a factor of 2 between the first and 14th daily doses, followed by a decline, as observed for other tissues. Although the pineal body was not detected in sections for females, the concentrations in this tissue matched those in the pituitary in both sexes. The peak concentrations in these tissues were much lower than those observed in major organs, such as the liver, kidney, heart and lung (Okada, 1997).

In conclusion, lufenuron accumulates in fat, from which it is slowly released into faeces, with terminal half-lives in fat of 5–13 days at 0.5 mg/kg bw and 10–37 days at 100 mg/kg bw. This accumulation has to be taken into account when determining the oral absorption, as part of the faeces measured at 7–21 days after dosing will consist of the initially absorbed amount from tissues/carcass.

The oral absorption after a single low dose (0.1–0.5 mg/kg bw) is at least 70%, based on the ratio of AUC between oral and intravenous doses (Booth, 2004). This was confirmed by Hassler (2003), who found an oral absorption of around 80%, based on the concentration in tissues/carcass 1 day after a single dose of 0.5 mg/kg bw.

There were no studies with tissue/carcass measurements at 1 day to determine the oral absorption after a single high dose. Taking an average half-life of 14 days in carcass and tissues, the tissue/carcass concentrations at 21 days (Booth, 2004) or 7 days (Bissig, 1990) after a single high dose of 100 mg/kg bw would indicate an initial tissue/carcass concentration of around 15–20% of the administered dose. The faecal excretion in these studies at 1 day after dosing was around 70–80%. This figure also indicates that the oral absorption after a single high dose of 100 mg/kg bw would be around 20%.

Dogs

Maurer & Hotz (1999) studied the bioavailability and mass balance of [^{14}C] lufenuron (nominal specific radioactivity of 0.1 MBq/mg test compound) in beagle dogs. Immediately after feeding, a male and female were treated orally with a 10 mg/kg bw dose of the test compound (dissolved in PEG 300) via gelatine capsule. One more dog/sex received a 2.5 mg/kg bw intravenous dose prior to feeding, formulated in 5% Tween 80 in Solketal. During the subsequent 21 days, venous blood samples, urine and faeces were collected for measurement of radioactivity level (by sample oxidizer/β-counter) and the concentrations of

racemic lufenuron and metabolites (by high-performance liquid chromatography [HPLC] in blood and thin-layer chromatography [TLC] in excreta). The orally dosed dogs were sacrificed on day 21 and the concentrations of radiolabel and lufenuron/metabolites in the brain, liver, spleen, heart, lungs, skeletal muscle, skin and the subcutaneous, kidney and omental fat were measured as described. The distribution of radioactivity in skin samples was visualized by radioluminography. Standard haematology and clinical chemistry parameters were analysed in blood taken from the intravenously dosed dogs prior to treatment and at termination.

The orally dosed dogs showed no clinical signs after treatment. By contrast, the intravenously treated animals displayed periodic head shaking and reddened ears and gingiva for some minutes after dosing, and were "tired" over the next few hours. Nevertheless, all animals subsequently appeared to be in normal health and there were no observable effects on haematological or clinical biochemical parameters on day 21.

Oral absorption of lufenuron into the circulation was moderately rapid, with a mean T_{max} in blood of 4 hours which was followed by a 24- to 48-hour distribution phase. The apparent volume of distribution was 19 L/kg. Lufenuron was eliminated very slowly, with a mean residence time of 26 days and a half-life of approximately 20 days in blood. The oral bioavailability of lufenuron was estimated at 70%, based on comparison of AUCs in the blood of orally and intravenously treated animals until day 21. Up to 54% of the orally administered dose was excreted via the faeces (mainly during the first 24 to 72 hours), but only a minor portion (0.8–2.2%) was eliminated in the urine. Faecal radioactivity shed within 24 hours of administration consisted of unmetabolized lufenuron, but increasing quantities of one unidentified major metabolite and three minor components appeared over the second and third days.

Markedly less of the administered radioactivity was recovered from the female dog than the male (75% of dose equivalents versus 91%), which the study authors attributed to incomplete homogenization of the skin and muscle prior to radioanalysis. At termination, the male dog had retained 16% of administered radioactivity equivalents in the skin (mostly in the subcutis), 11% in the skeletal muscle, 7% in adipose tissue, 1% in the liver and bile combined, and 0.3% in the blood.

2.1.2 Biotransformation

A single major component, parent lufenuron, was identified in faecal extracts after single oral doses of lufenuron at 0.1, 1.0, 10 or 100 mg/kg bw. The levels of administered radioactivity in urine were very low (<1% of the dose), and metabolite identification was not performed on these samples (Booth, 2004; see section 1.1).

In Hassler (2003), described in section 1.1, chromatographic analysis of rat urine revealed a metabolite profile comprising at least 10 metabolite fractions. Although the total amount of radiolabelled metabolites was very low, based on very low urinary excretion, the patterns were qualitatively and quantitatively similar after single and repeated dosing.

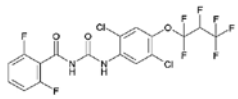
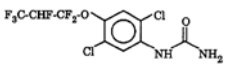
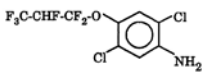
TLC analysis of the faecal extracts revealed at least four metabolite fractions. The extractability and the metabolite pattern of the faeces were essentially identical for all three selected sampling intervals, with slight quantitative variations mainly between the first time interval (0–1 day) and the later selected time intervals. The major metabolite fraction (3) corresponded to unchanged lufenuron, accounting for approximately 30% of the daily dose. Apart from unchanged parent, only minor metabolites were present in the faecal extracts (Hassler, 2003).

In Okada (1997), described in section 1.1, chromatographic analysis of metabolite profiles in plasma and cerebrum revealed the following: In plasma, 96% of the radioactivity was extracted into methanol and was resolved into four metabolites. The major component was unchanged lufenuron; another major metabolite was CGA 238277. One of the minor metabolites corresponded to CGA 224443, and the other was unidentified (Table 7). The proportion of lufenuron in all samples was approximately 70–80% and appeared to be unaffected by the number of doses given or the sampling time. More than 94% of radioactivity in the cerebrum was extracted and comprised only unchanged lufenuron, representing more than 92%, and CGA 238277, accounting for 1.1% or less of the radioactivity (Okada, 1997).

In another study described in section 1.1 (Bissig, 1990), the urinary metabolite pattern after a single dose of either 0.5 or 100 mg/kg bw was not determined because of the low recovery of radioactivity in urine (<1% of the dose). Analysis of faeces by one-dimensional TLC revealed no significant differences between the sexes and no significant influence of pretreatment with unlabelled lufenuron, but showed slightly dose-dependent differences. The metabolite pattern consisted of one dominant fraction (F3), which was characterized as unchanged lufenuron. As a consequence of the higher amount of lufenuron excreted in faeces at the high dose, metabolite fraction F3 represented a higher percentage of the dose (77–79%) than at the low dose level (37–48%). In contrast, the most polar fractions (F1 and F2) were more pronounced at the low dose than at the high dose.

The faecal metabolite pattern of the bile duct–cannulated rats (group G) was similar to that of the animals of group B, but with a higher percentage of unchanged lufenuron. Radioactivity accounted for only 1.5% of the administered dose. Metabolite fraction G5, which represented 0.1% of the administered dose, co-chromatographed with unchanged lufenuron. The metabolite fraction G2

Table 7
Summary of lufenuron-derived metabolites in the rat

Metabolite code	Chemical name	Structure
Lufenuron, (CGA 184699)	(*RS*)-1-[2,5-Dichloro-4-(1,1,2,3,3,3-hexafluoropropoxy)phenyl]-3-(2,6-difluorobenzoyl)urea	
CGA 238277	1-[2,5-Dichloro-4-(1,1,2,3,3,3-hexafluoropropoxy)phenyl]-urea	
CGA 224443	2,5-Dichloro-4-(1,1,2,3,3,3-hexafluoropropoxy)aniline	

Source: Thanei (1990)

(corresponding to 0.1% of the dose) behaved as the authentic reference compound CGA 238277 (i.e. the free urea conjugate of the dichlorophenyl moiety). The least polar metabolite fraction G6 co-chromatographed with CGA 224443, the free amine derivative of the dichlorophenyl moiety (<0.1% of the dose).

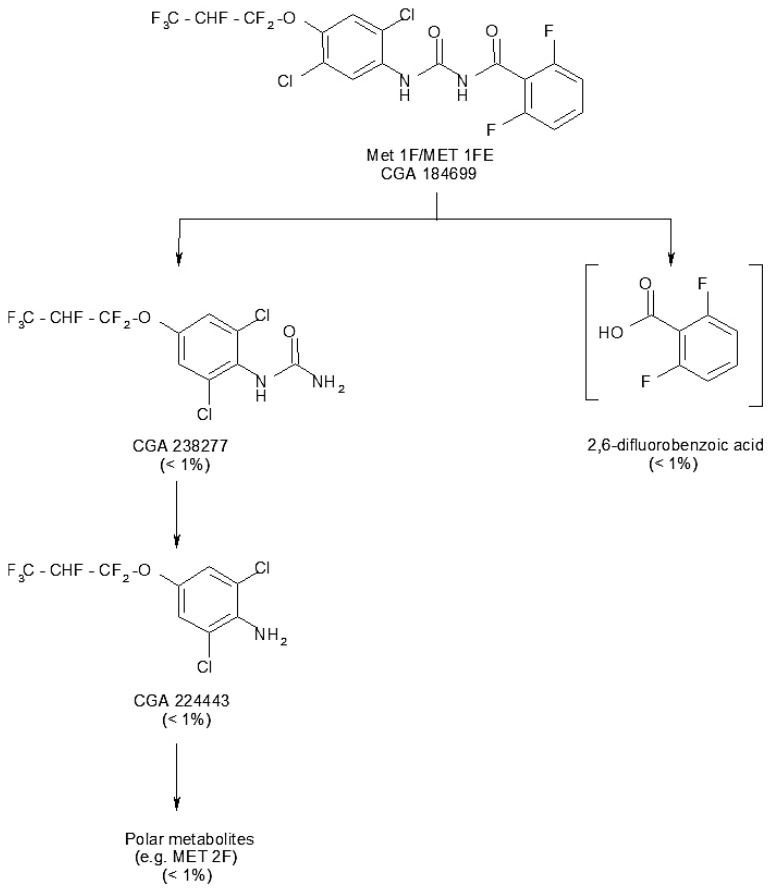
Samples of fat, liver, kidneys, lungs and carcass were pooled by sex and group to analyse ^{14}C residues. TLC analysis of organic solvent and aqueous extracts of tissues revealed a single metabolite fraction in all extracts, co-chromatographing with unchanged lufenuron (Bissig, 1990).

To further investigate the metabolism of lufenuron after a single oral administration, samples used in this study originated from a study described above (Bissig, 1990). Faeces and fat collected from male and female rats administered a single oral dose of [^{14}C]lufenuron at 100 mg/kg bw were used in the present study.

The 8- to 48-hour faecal pools (representing 76% of the dose) for male and female rats were extracted with methanol. Almost 100% of the radioactivity in faeces was extractable. The TLC analysis revealed one dominant metabolite fraction, accounting for 75% of the dose. This major metabolite fraction was identified by spectroscopic means to be unchanged lufenuron. The minor metabolite fractions accounted for less than 1% of the dose each. Lufenuron was metabolized to a very small extent. In addition, two metabolites were characterized by chromatographic comparison with authentic reference compounds (Table 7).

Organic solvent extraction of the single pool of fat from male and female rats extracted approximately 93% of the radioactivity present. Purification by preparative TLC yielded two fractions. The fraction containing almost all the radioactivity was further purified by HPLC. It was shown by mass spectroscopy and by co-chromatography to be identical to the parent compound, lufenuron. By chromatographic comparison, the second fraction corresponded to metabolite F2.

Fig. 3
Proposed metabolic pathway for lufenuron in the rat



Source: Thanei (1990)

Mostly unchanged lufenuron was excreted in faeces and was also retained in tissues, predominantly in fat, demonstrating that lufenuron was poorly metabolized. It is assumed that a minor route of degradation is cleavage of the benzamide moiety, yielding the urea CGA 238277 and 2,6-difluorobenzoic acid. Further cleavage of the ureido moiety of CGA 238277 leads to the aniline CGA 224443. It is established that rats excrete 2,6-difluorobenzoic acid largely unchanged. Based on the faecal and fat metabolites identified or characterized, which represented approximately 85% of the dose, the proposed metabolic pathway in the rat is summarized in Fig. 3 (Thanei, 1990).

Table 8
Acute toxicity of lufenuron and two soil metabolites

Species	Strain	Sex	Route	Purity (%)	Result	Reference
Lufenuron						
Mouse	Tif: MAGf (SPF)	M + F	Oral	95.2	LD_{50} > 2 000 mg/kg bw	Hartmann (1989)
Rat	Tif: RAIf (SPF)	M + F	Oral	95.2	LD_{50} > 2 000 mg/kg bw	Hartmann (1988a)
Rat	Tif: RAIf (SPF)	M + F	Dermal	95.2	LD_{50} > 2 000 mg/kg bw	Hartmann (1988b)
Rat	Tif: RAIf (SPF)	M + F	Inhalation	95.2	LC_{50} > 2.35 mg/L (maximal attainable concentration)	Hartmann (1988c)
CGA 149772						
Rat	Tif: RAIf (SPF)	M + F	Oral	98.2	LD_{50} > 1 000 mg/kg bw in males (highest tested dose) LD_{50} = 1 836 mg/kg bw in females	Hartmann (1988d)
Rat	Tif: RAIf (SPF)	M + F	Dermal	98.2	LD_{50} > 2 000 mg/kg bw	Hartmann (1988e)
Rat	Tif: RAIf (SPF)	M + F	Inhalational	95.2	LC_{50} > 5.3 mg/L	Hartmann (1988f)
CGA 224443						
Rat	Tif: RAIf (SPF)	M + F	Dermal	92.6	LD_{50} > 4 000 mg/kg bw	Hartmann (1990a)
Rat	Tif: RAIf (SPF)	M + F	Inhalational	92.6	LC_{50} > 5.1 mg/L	Hartmann (1990b)

bw: body weight; F: female; LC_{50}: median lethal concentration; LD_{50}: median lethal dose; M: male

2.2 Toxicological studies

2.2.1 Acute toxicity studies

(a) Lethal doses

The results of acute toxicity studies with lufenuron and the two soil metabolites, CGA 149772 and CGA 224443 administered orally, dermally or by inhalation are summarized in Table 8. All the studies were certified to comply with GLP and performed according to United States Environmental Protection Agency and OECD guidelines.

Lufenuron has low acute toxicity when administered orally, dermally and via inhalation to rats.

Lufenuron

In an acute oral toxicity study, Tif: MAGf (SPF) mice (5/sex per group) received an oral dose of lufenuron (purity 95.2%) at 2000 mg/kg bw. The test material was suspended in Oleum arachidis Ph.H.VI. There were no deaths. Clinical signs were slight and limited to piloerection, hunched posture and dyspnoea. The animals recovered within 6 days. Body-weight gains were normal. There were no gross necropsy findings. The LD_{50} following oral exposure was greater than 2000 mg/kg bw (Hartmann, 1989).

In an acute oral toxicity study, Tif: RAIf (SPF) rats (5/sex per group) received an oral dose of lufenuron (purity 95.2%) of 2000 mg/kg bw. The test was

suspended in Oleum arachidis Ph.H.VI. There were no deaths, and clinical signs were limited to ruffled fur, dyspnoea, hunched posture and exophthalmos, ranging from moderate directly after treatment to slight up to 10 days after treatment. Body-weight gains were normal. There were no gross necropsy findings. The LD_{50} following oral exposure was greater than 2000 mg/kg bw (Hartmann, 1988a).

In an acute dermal toxicity study, Tif: RAIf (SPF) rats (5/sex per group) were treated with lufenuron (purity 95.2%) via dermal exposure at a limit dose of 2000 mg/kg bw. The test material was suspended in Oleum arachidis Ph.H.VI and administered in 0.4 mL/kg bw to approximately 10% of the body surface area under an occlusive dressing for 24 hours. There were no deaths. The animals showed ruffled fur, dyspnoea, abnormal body positions and reduced spontaneous activity. Clinical signs were slight; the animals recovered within 5 days. No skin irritation was noted. Body-weight gains were normal. There were no gross necropsy findings. The LD_{50} following dermal exposure was greater than 2000 mg/kg bw (Hartmann, 1988b).

In an acute inhalation toxicity study, Tif: RAIf (SPF) rats (5/sex per group) were exposed to lufenuron (purity 95.2%) for 4 hours via nose-only exposure at a test atmosphere concentration of 2.35 mg/L (nominal 3.0 mg/L, mass median aerodynamic diameter [MMAD] 1.9–2.8 μm, geometric standard deviation [GSD] 2.2–2.5 μm). There were no deaths. Clinical signs recorded during exposure were moderate piloerection, slight hunched posture and moderate dyspnoea. All animals recovered within 2 days. Males exhibited a significantly higher body-weight increase during the second week after exposure. There were no notable necropsy findings at termination. The acute inhalation median lethal concentration (LC_{50}) was greater than 2.35 mg/L (Hartmann, 1988c).

Metabolite CGA 149772

In an acute oral toxicity study, Tif: RAIf (SPF) rats (5/sex per group) received metabolite CGA 149772 (purity 98.2%) at a dose of 500 or 1000 mg/kg bw in males and 1000 or 2000 mg/kg bw in females. The test article was suspended in 0.5% (weight per weight [w/w]) carboxymethylcellulose and 0.1% (w/w) polysorbate 80. Three females at 2000 mg/kg bw died. Clinical signs were moderate, being limited to ruffled fur, dyspnoea, hunched posture and exophthalmos, from directly after treatment for about 5 days. There were no gross necropsy findings in male rats, but oedematous and haemorrhagic lungs were observed in the females that died. The LD_{50} was greater than 1000 mg/kg bw in males and 1836 mg/kg bw in females (Hartmann, 1988d).

In an acute dermal toxicity study, Tif: RAIf (SPF) rats (5/sex per group) were treated with metabolite CGA 149772 (purity 98.2%) at a limit dose of 2000 mg/kg bw. The test article was suspended in 0.5% (w/w) carboxymethylcellulose

and 0.1% (w/w) polysorbate 80 and 4 mL/kg bw was applied to approximately 10% of the body surface area and kept under an occlusive dressing for 24 hours. There were no deaths. Clinical signs were ruffled fur, dyspnoea, abnormal body positions and reduced spontaneous activity. The clinical signs were slight, and animals recovered within 9 days. No skin irritation was noted. There were no gross necropsy findings. The LD_{50} following dermal exposure was greater than 2000 mg/kg bw (Hartmann, 1988e).

In an acute inhalation toxicity study, Tif: RAIf (SPF) rats (5/sex per group) were exposed to metabolite CGA 149772 (purity 98.2%) for 4 hours via nose-only exposure at a test atmosphere concentration of 5.3 mg/L (MMAD 1.4–1.6 μm, GSD 1.4–1.5 μm). There were no deaths, and clinical signs recorded during exposure were moderate piloerection, slight hunched posture and moderate dyspnoea. All animals recovered within 3 days. There were no notable necropsy findings at termination. The acute inhalation LC_{50} was greater than 5.3 mg/L (Hartmann, 1988f).

Metabolite CGA 224443

In an acute dermal toxicity study, Tif: RAIf (SPF) rats (5/sex per group) were treated with metabolite CGA 224443 (purity 92.6%) at a dose of 4000 mg/kg bw. The liquid test article was applied undiluted to approximately 10% of the body surface area and kept under an occlusive dressing for 24 hours. There were no deaths. Clinical signs were ruffled fur, dyspnoea, abnormal body positions and reduced spontaneous activity. The clinical signs were slight, and animals recovered within 5 days. No skin irritation was noted. There were no gross necropsy findings. The LD_{50} following dermal exposure was greater than 4000 mg/kg bw (Hartmann, 1990a).

In an acute inhalation toxicity study, Tif: RAIf (SPF) rats (5/sex per group) were exposed to metabolite CGA 224443 (purity 92.6%) for 4 hours via nose-only exposure at a test atmosphere concentration of 5.1 mg/L (MMAD 2.4–3.5 μm, GSD 1.6–1.7 μm). There were no deaths, and clinical signs recorded during exposure were moderate piloerection, slight hunched posture and dyspnoea. All animals exposed to 2.02 mg/L recovered within 13 days, but clinical signs persisted until the end of the 14-day observation period in rats exposed to 5.1 mg/L. One rat at 2.08 mg/L died on day 2 after exposure and three at 5.1 mg/L died on days 3, 10 and 13 days after exposure. However, a histopathological examination of the lungs suggested that the rats had died of primary obstruction of the airways that was not treatment related. There were no notable necropsy findings in survivors at scheduled kill. The acute inhalation LC_{50} was greater than 5.1 mg/L (Hartmann, 1990b).

(b) **Dermal irritation**

In a skin irritation study, three female New Zealand White (KFM-NZW) rabbits were treated with lufenuron (purity 95.2%) via dermal exposure at a dose of 0.5 g applied to the flank under an occlusive dressing for 4 hours. The (solid) test material was applied to the skin using a gauze patch that was moistened before application with distilled water containing 0.5% carboxymethylcellulose and 0.1% polysorbate 80. Very slight erythema was observed in two animals at 1 hour after removal of the test substance. No other skin reactions were observed in any animal during the study. Body-weight gains were normal (Schneider, 1988a).

(c) **Ocular irritation**

In an eye irritation study, three male New Zealand White (KFM-NZW) rabbits were treated with lufenuron (purity 95.2%) via ocular exposure at a dose of 0.1 mL (70 mg). Slight irritation of the cornea, iris and conjunctiva was observed at 1 hour after instillation in all three animals. Slight conjunctival redness was also found in one male 24 hours after instillation. No signs of eye irritation were present at the 48- and 72-hour examinations (Schneider, 1988b).

(d) **Dermal sensitization**

In a Magnusson and Kligman maximization test, lufenuron (purity 95.2%) was tested using 10 male and 10 female Pirbright White Strain (Tif: DHP) guinea-pigs in the test substance group and five male and five female guinea-pigs in the control group. No results of the intradermal injection/topical induction are presented. Following challenge with lufenuron in petrolatum at 10% weight per weight (w/w), dermal responses were observed in 4/20 test animals at 24 hours after challenge and in 9/20 test animals at 48 hours after challenge. No skin reactions were observed in control animals. Up to 45% of the animals were sensitized by lufenuron under the experimental conditions employed. Therefore, lufenuron is considered to be a skin sensitizer in the guinea-pig. Sensitization of this strain of animals was positively tested with dinitrochlorobenzene (positive control), which gave very severe allergic reactions in all areas treated (Schneider, 1988c).

2.2.2 Short-term studies of toxicity

(a) **Oral administration**

(i) Lufenuron

Mice

In a dose range–finding study, lufenuron (purity 95.2%) was fed to mice (Tif: MAGf (SPF), hybrids of NIH × MAG; 10/sex per group) at a concentration of

0, 1000, 3000 or 9000 ppm (mean substance intakes: 0, 151, 449 and 1470 mg/kg bw per day for males and 0, 189, 517 and 1440 mg/kg bw per day for females, respectively) for up to 65 days. No haematological examinations were carried out, and no organ weights were measured.

As a result of high mortality and neurotoxic effects (tonic–clonic seizures) of the test substance, the surviving animals at 3000 and 9000 ppm were euthanized on day 49. Eleven animals (four males and seven females) treated at 3000 ppm and 19 animals (nine males and 10 females) treated at 9000 ppm were found dead between days 17 and 48. At 1000 ppm, there was a slight increase in mortality (one male and three females died between days 54 and 65). Tonic–clonic seizures were also observed in two animals at 1000 ppm. A small reduction in body-weight gain and decreased feed consumption were observed in all treated males. It was concluded that the maximum tolerated dose (MTD) was exceeded even at 1000 ppm (equal to 151 and 189 mg/kg bw per day for males and females, respectively) (Fankhauser, 1989a).

In a second dose range–finding study intended to determine test substance residue and blood levels, lufenuron (purity 95.2%) was fed to groups of 30 female mice (Tif: MAGf (SPF), hybrids of NIH × MAG) at a concentration of 0, 4/8, 20, 100 or 1000 ppm (mean substance intakes: 0, 0.47/1.1, 2.94, 14.5 and 143 mg/kg bw per day, respectively) for up to 91 days (only 71 days for the high dose). From day 57 onwards, the diet of the low-dose animals inadvertently contained 8 ppm instead of 4 ppm. Subgroups of nine mice were killed at weeks 9, 11 and 14; the remaining three animals per dose group were intended for eventual replacement or pharmacological investigations. Fat and blood samples were collected at 9 and 11 weeks. Brain samples were collected at terminal kill at week 14. No haematological or clinical chemistry examinations were carried out, and no organ weights were measured.

There was a significant increase in mortality (eight animals) and signs of neurotoxicity (tonic–clonic seizures in four animals) at 1000 ppm. Therefore, the remaining animals at 1000 ppm (six from the last sacrifice group and the replacement group) were killed on day 71. No effects on body weight or feed consumption were observed, and there were no macroscopic findings. Concentrations of lufenuron in blood, fat and brain were dose dependent, with equilibrium reached by week 9 of the study. Lufenuron concentrations in fat were approximately 100-fold those in blood and brain (Table 9).

The NOAEL after 90 days in this dose range–finding study in female mice was 100 ppm (equal to 14.5 mg/kg bw per day), based on mortality and neurotoxicity at 1000 ppm (equal to 143 mg/kg bw per day) (Fankhauser, 1990).

Table 9
Blood, fat and brain concentrations of lufenuron observed in a 3-month range-finding study in female mice given lufenuron in the diet

Dietary concentration	Blood (mg/L)			Fat (mg/kg)			Brain (mg/kg)
(ppm)	Week 9	Week 11	Week 14	Week 9	Week 11	Week 14	Week 14
0	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.08
4/8	0.13	0.36	0.52	10.8	29.9	49.9	0.41
20	0.87	0.9	1.45	81.7	76.5	81.6	0.65
100	5.02	5.24	5.05	488.7	499.5	487.8	5.19
1 000	48.5	43.4	n.a.	4 537.7	5 400 (all survivors)	n.a.	187 (week 11)

n.a.: not applicable, as this dose group was terminated in week 11; ppm: parts per million
Source: Fankhauser (1990)

Rats

In a 28-day range-finding study, groups of five male and five female Tif: RAIf (SPF) rats were administered lufenuron (purity 95.2%) in the diet at a concentration of 0, 50, 400, 3000 or 20 000 ppm (mean substance intakes: 0, 4.10, 30.8, 254 and 1692 mg/kg bw per day for males and 0, 4.07, 32.6, 254 and 1741 mg/kg bw per day for females, respectively). Investigations included clinical signs, body weights, feed consumption, haematology and clinical chemistry at termination, urine analysis, organ weights, gross necropsy and histopathological examination.

No mortality and no treatment-related clinical findings were observed. At weeks 3 and 4, the mean body-weight gain and feed consumption of males fed 20 000 ppm lufenuron were reduced, not statistically significantly, by about 13% and 12–16%, respectively. Feed utilization values were not affected in any group. There were no statistically significant differences in water consumption between the groups.

There were no treatment-related differences between the groups in haematological or blood clinical chemistry parameters; some minor differences did achieve statistical significance, but these were representative of the normal variation of the respective parameters. No treatment-related differences were observed in urinary clinical chemistry parameters.

There were no treatment-related differences in macroscopic findings postmortem. After exsanguination, the mean body weight of males fed 20 000 ppm lufenuron was about 15% lower than that of the control group. The increased mean testis-to-body-weight ratio and decreased liver weights seen in these males were therefore considered unrelated to treatment. Absolute and relative thymus weights of animals fed 3000 or 20 000 ppm lufenuron were reduced by about

Table 10
Intergroup comparison of selected organ weights

	Absolute/relative (to body weight) organ weights									
	Males					Females				
Organ	0 ppm	50 ppm	400 ppm	3 000 ppm	20 000 ppm	0 ppm	50 ppm	400 ppm	3 000 ppm	20 000 ppm
Thymus										
Absolute (mg)										
Mean	792.9	749.5	725.0*	597.5	556.1	524.2	434.8	455.1	405.0	393.9
SD	239.9	103.3	89.22	116.7	110.6	214.1	106.7	67.25	69.45	59.65
Min.	530.5	639.8	637.9	471.2	447.5	338.7	313.8	354.9	316.4	332.4
Max.	1 086	872.4	836.2	752.7	733.6	799.4	601.6	536.5	492.7	492.1
Relative (%)										
Mean	2.530	2.451	2.337*	2.008	2.091	2.665	2.311	2.309	2.332*	2.109
SD	0.595	0.409	0.257	0.343	0.287	0.884	0.544	0.311	0.266	0.326
Min.	1.882	1.919	1.993	1.660	1.792	1.933	1.616	1.767	2.105	1.781
Max.	3.104	2.848	2.607	2.514	2.568	3.863	3.096	2.535	2.678	2.622
Ovary/testis										
Absolute (mg)	3.305	3.573	3.430	3.382	3.517	171.3	161.0	166.7	158.5	192.8
Relative (%)	10.68	11.63	11.08*	11.41	13.29*	0.886	0.856	0.844	0.912	1.031
Liver										
Absolute (mg)	14.95	15.00	15.69	15.44	13.44	8.309	7.941	9.454	8.182	8.408
Relative (%)	48.30	48.61	50.32	51.96	50.74	43.13	42.16	48.02	47.25	44.92

Max.: maximum; Min.: minimum; ppm: parts per million; SD: standard deviation; *: $P < 0.05$
Source: Fankhauser (1988)

13–30% compared with controls. The thymus weight of 400 ppm males was only slightly reduced (9%), and the statistical significance was due to a rather low standard deviation at 400 ppm compared with the one for controls. Moreover, the thymus weight at 400 ppm in males was within the range of controls. The reduced thymus weight at 400 ppm was therefore not considered to be adverse. In females fed 20 000 ppm lufenuron, slightly increased ovary weights were noted (Table 10). There were no treatment-related changes seen during histopathological examination.

The NOAEL for lufenuron when fed to rats continuously for a 28-day period was 400 ppm (equal to 30.8 and 32.6 mg/kg bw per day for males and females, respectively), based on decreased thymus weight at 3000 ppm (equal to 254 mg/kg bw per day for both males and females) (Fankhauser, 1988).

In a 90-day oral toxicity study, groups of 20 male and 20 female Tif: RAIf (SPF) rats were fed diets containing lufenuron (purity 95.2%) at 0 or 15 000 ppm, for 92 or 93 consecutive days. Additional groups of 10 male and 10 female rats were fed diets containing lufenuron (purity 95.2%) at 25, 150 or 1500 ppm, for

92 or 93 consecutive days. Mean substance intakes were 0, 1.6, 9.68, 101 and 998 mg/kg bw per day for males and 0, 1.7, 10.2, 103 and 1050 mg/kg bw per day for females at 0, 25, 150, 1500 and 15 000 ppm, respectively. In each dose group, 10 animals/sex per group were killed at the end of the treatment period. In the control and highest dose groups, 10 animals/sex were kept for a 4-week recovery period before scheduled kill. Clinical observations, body weights and feed and water consumption were measured throughout the study. Ophthalmological examinations of control and highest-dose animals were conducted pre-study and towards the end of the treatment and recovery periods. Haematology and blood chemistry analyses were carried out on all animals towards the end of the treatment period. At the end of the scheduled period, all animals were killed and examined postmortem, selected organs were weighed and specified tissues were taken for subsequent histopathology.

One female fed 15 000 ppm lufenuron was found dead on day 98, after showing signs of emaciated condition and motor disorder. There were no other treatment-related deaths. Tonic–clonic seizures were observed in 9/20 males and 8/20 females fed 15 000 ppm lufenuron and in 1/10 females fed 1500 ppm lufenuron. Two males still exhibited tonic–clonic seizures in the last week of the recovery period. There was no evidence of any effect of treatment on the eyes. The mean body-weight gain of males from groups fed 1500 or 15 000 ppm lufenuron was decreased from week 2 until the end of treatment. At 15 000 ppm lufenuron, mean body weights comparable with control values were reached at the end of the recovery period. A similar tendency was seen in females at these dose levels. During the treatment period, slightly, but not statistically significantly, reduced feed consumption was seen in animals fed 1500 or 15 000 ppm lufenuron. A compensatory increase was seen in the animals retained for the recovery period. Water consumption was not affected in any group.

Haematocrit values and prothrombin time were increased slightly at the end of treatment in females fed 1500 and 15 000 ppm lufenuron, but were reversible within the recovery period. White blood cell counts in females fed 15 000 ppm lufenuron were at the upper end of the normal range at week 14 and at the end of the recovery period. Some changes in plasma phosphate and sodium concentrations and in alkaline phosphatase (ALP) activity were seen in male and/or female animals fed 1500 or 15 000 ppm lufenuron; these changes were not reversible over the recovery period. Other minor changes in blood chemistry, which were reversible, were seen in cholesterol, albumin, protein and chloride levels and decreased albumin-to-globulin ratios. Alanine aminotransferase (ALAT) and ALP activities were slightly higher in females fed 15 000 ppm lufenuron. Other haematological and clinical chemistry parameters that achieved a statistically significant difference from control values were not biologically significant.

Table 11
Fat concentrations of lufenuron in rats after a 3-month treatment

	Concentrations of lufenuron (mg/kg fat)				
Sex	**0 ppm**	**25 ppm**	**150 ppm**	**1 500 ppm**	**15 000 ppm**
Males	3.2[a]	47.2	378	3 025	2 710
Females	4.4[a]	44	447	3 875	3 176

ppm: parts per million
[a] Most probably due to contamination of samples.
Source: Fankhauser (1989b)

There were no treatment-related changes in macroscopic findings postmortem. Absolute and relative liver weights of females fed 15 000 ppm lufenuron were increased by 13–16% and 20–32% at the end of the treatment period and recovery period, respectively. In males at this dose, the ratio was increased by 13% at the end of the treatment period, with values comparable with control values after the recovery period. Adrenal weights in both sexes at this dose level were increased by 20–36%; in females at 1500 ppm lufenuron, they were increased by 11–16%. Adrenal weights were comparable with control values at all doses at the end of the recovery period. Other findings in organ weights that achieved a statistically significant difference from control values were not biologically significant. There were no treatment-related changes in microscopic findings postmortem.

In order to support the selection of dose levels for a 2-year toxicity and oncogenicity study and to study the accumulation of lufenuron in fat tissues, fat samples were collected at terminal kill at week 14. The content of lufenuron in fat increased dose-dependently at 25, 150 and 1500 ppm. The concentrations were similar at 1500 and 15 000 ppm, indicating that a solubility limit in fat had been reached (Table 11). Fat tissue concentrations were similar in males and females.

Some of the control samples contained traces of the test article. Reconstruction of the sampling procedure and a confirmational analysis of leftover samples indicated that this was most probably due to contamination of the samples rather than a feeding error. The interpretation of the analytical results was not considered to be compromised by this finding.

The NOAEL for lufenuron in rats when fed continuously in the diet for a period of up to 3 months was 150 ppm (equal to 9.68 and 10.2 mg/kg bw per day for male and female animals, respectively), based on clinical signs (tonic–clonic seizures), decreased body-weight gain and feed consumption, slight changes in haematology and clinical chemistry parameters, and increased adrenal weights at 1500 ppm (equal to 101 and 103 mg/kg bw per day for male and female animals, respectively) (Fankhauser, 1989b).

Dogs

In a 4-week range-finding study, groups of two male and two female beagle dogs received lufenuron (purity 95.2%) in their diet at a concentration of 200 or 50 000 ppm (equal to 8.43 and 2200 mg/kg bw per day for males and 10.1 and 2648 mg/kg bw per day for females, respectively). Blood samples were taken pretest and at intervals during the study and were analysed for proof of absorption. All the dogs were examined daily for clinical condition and mortality. Detailed clinical examinations were conducted and body weights and feed consumption were measured or recorded at intervals during the study. Haematological and clinical chemistry examinations of blood were performed pretest and after 2 and 4 weeks. At the end of the study, all the animals were killed and macroscopically examined. Final body weights and selected organ weights were recorded. A comprehensive range of tissues was examined histopathologically.

There were no deaths, and no clinical signs of toxicity were seen during the study. No abnormalities were detected during the detailed clinical and physical examinations. There was no effect on body weight or feed consumption. There were no toxicologically significant changes in any of the haematological or clinical chemistry parameters examined. No macroscopic or microscopic abnormalities were detected, and there was no effect on organ weights. The mean levels of lufenuron found in the blood were 4 and 16 ppm, and those in the fat were 615 and 2750 ppm, at the low and high doses, respectively.

The NOAEL was 50 000 ppm (equal to 2200 and 2648 mg/kg bw per day for males and females, respectively), the highest dose tested (Briffaux, 1989a).

In another 4-week range-finding study, groups of two male and two female beagle dogs received an oral dose of 50 mg/kg bw per day of a water-based veterinary preparation containing 15% lufenuron. According to the report, this was 5 times the anticipated therapeutic dose. No further information on the test formulation was provided in the report; hence, the exact composition remains unclear. The following information was provided in the European Union Draft Assessment Report (Portugal, 2006):

> The Notifier informed that the test formulation used was a pilot formulation for a commercial veterinary product whose recipe is proprietary information now belonging to another company. The Notifier assumed that the main component is water, with the addition of glycerine and avicel (a mixture of microcrystalline cellulose and carboxymethylcellulose sodium, which acts as a water insoluble dispersible colloidal excipient) and on this basis found it reasonable to believe that the toxicity seen in this study is due to the 15% lufenuron in the formulation, and not to any of the formulation additives.

All the dogs were examined daily for clinical condition and mortality. Detailed clinical examination, ophthalmology, body weights and feed consumption were measured or recorded at intervals during the study. Haematological and clinical chemistry examinations of blood were performed pretest and after 2 and 4 weeks. Blood samples were taken at intervals and analysed for lufenuron concentration. At the end of the study, all the animals were killed humanely and given a macroscopic examination postmortem. Final body weights and selected organ weights were recorded. Tissues were not examined histopathologically.

The levels of lufenuron in blood showed a steady increase during the 4-week study. After 1 week, they ranged from 0.57 to 7.51 μg/mL; after 2 weeks, from 1.63 to 9.80 μg/mL; and after 4 weeks, from 2.46 to 16.0 μg/mL. There were no deaths, and no clinical signs of toxicity were seen during the study. No abnormalities were detected during the detailed clinical and physical examinations, including ophthalmology. There was no effect on body weight or feed consumption. Haematological examination showed that platelet counts were slightly elevated in the males at 2 and 4 weeks compared with the pretest values. No other haematological or blood chemistry changes were detected. No significant macroscopic abnormalities were seen, and there was no effect on organ weights.

In this 4-week range-finding study in dogs, the NOAEL of a veterinary lufenuron formulation with 15% active substance was 50 mg/kg bw per day (corresponding to a lufenuron dose of 7.5 mg/kg bw per day), the only dose tested (Pavkov & Macaskill, 1989).

In a 90-day toxicity study, beagle dogs (4/sex per group) received lufenuron (purity 95.2%) in their diet at a concentration of 0, 200, 3000 or 50 000 ppm (equal to 0, 7.8, 121.6 and 2023 mg/kg bw per day for males and 0, 7.9, 122.5 and 1933 mg/kg bw per day for females, respectively). An additional two animals/sex in the control and high-dose groups were given a 4-week recovery period after the 90-day dosing period. Clinical observations (including ophthalmoscopy), body weights, feed consumption, haematology and clinical biochemistry were measured throughout the study. At the end of the scheduled periods, the animals were killed and examined postmortem. Bone marrow smears were taken, selected organs were weighed and specified tissues were taken for subsequent histopathological examination.

There were no mortalities. No treatment-related clinical or ophthalmological findings were recorded. Feed consumption and body weight were not affected by treatment. No changes in haematology parameters attributable to a toxic effect of the compound were observed. A slight decrease in blood phosphorus level was observed at 3000 and 50 000 ppm in both sexes at week 6 and in females at week 13. At week 17 (end of the recovery period), mean blood phosphorus levels were similar for control and 50 000 ppm animals. A

slight decrease in blood potassium level was observed at week 13 in the 3000 and 50 000 ppm females, without a dose–response relationship. At week 17, mean blood potassium levels were similar for control and 50 000 ppm animals. A moderate to marked increase in total blood cholesterol level was observed at weeks 6 and 13 for both sexes at 3000 and 50 000 ppm. A moderate increase was still present at week 17 in the 50 000 ppm males, although this was not statistically significant. A moderate increase in ALP activity was seen at weeks 6 and 13 for females at 200 and 3000 ppm and at week 13 for males at 3000 ppm, with no evidence of a clear dose–response relationship. At week 17, all individual ALP values were considered normal (Table 12). Statistically significant differences from controls were also seen in some glucose, albumin and transaminase values recorded, but these differences were considered not toxicologically significant and were not treatment related.

An apparent increase in the volume of urine associated with a decrease in specific gravity was seen at weeks 6, 13 and 17 in the 50 000 ppm males. When the values at pretest and in weeks 6, 13 and 17 were compared for individual dogs, no trends were evident. The semiquantitative estimations and the microscopic examination of the spun deposits in the urine did not reveal any treatment-related changes.

Absolute liver weights were increased at terminal kill at week 13 in both sexes at 3000 and 50 000 ppm (132% and 131% for males and 125% and 132% for females, respectively), reaching statistical significance only in males at 50 000 ppm. Liver to terminal body-weight ratios were increased statistically significantly at terminal kill at week 13 in both sexes at 3000 and 50 000 ppm (142% and 142% for males and 131% and 131% for females, respectively). Liver to brain-weight ratios were statistically significantly increased in males at 50 000 ppm (135%). At the end of the recovery period, there was some evidence of reversibility, with none of the increases being statistically significant (Table 12).

No gross or microscopic changes were observed that correlated with the increased liver weights observed in the dogs at 3000 or 50 000 ppm. There were no macroscopic or microscopic findings postmortem that could be attributed to treatment with lufenuron.

The NOAEL was 200 ppm (equal to 7.8 and 7.9 mg/kg bw per day for males and females, respectively), based on increased blood cholesterol levels and absolute and relative liver weights, reductions in blood potassium and phosphorus levels, and an increase in serum ALP activity for some animals at 3000 ppm (equal to 121.6 and 122.5 mg/kg bw per day for males and females, respectively) (Briffaux, 1989b).

In a 1-year toxicity study, groups of four male and four female beagle dogs received lufenuron (purity 95.3%) in their diet at a concentration of 0, 100, 2000 or 50 000 ppm (equal to 0, 3.97, 65.4 and 1879 mg/kg bw per day for males

Table 12

Intergroup comparison of blood biochemistry (selected parameters) and liver weights in a 90-day dog study[a]

		Males				Females			
Parameter	**Week**	**0 ppm**	**200 ppm**	**3 000 ppm**	**50 000 ppm**	**0 ppm**	**200 ppm**	**3 000 ppm**	**50 000 ppm**
Phosphorus (mg/L)	0	75	77	72	71	70	71	72	70
	6	67	67	58**	56***	59	60	52***	49***
	13	59	56	48	50	54	52	41*	44**
	17	50	–	–	53	47	–	–	46
Potassium (meq./L)	0	4.7	4.9	5.0	4.9	4.8	5.1	4.7	4.8
	6	4.6	4.9	4.6	4.7	4.8	5.1	4.7	4.8
	13	5.0	5.2	4.8	4.7	4.9	4.9	4.2*	4.3*
	17	4.3	–	–	4.7	4.7	–	–	4.5
Cholesterol (g/L)	0	1.37	1.28	1.36	1.39	1.23	1.29	1.43	1.30
	6	1.20	1.45	1.95*	2.22***	1.35	1.48	2.17*	2.13*
	13	1.20	1.42	1.86*	1.98**	1.40	1.43	2.42*	2.09*
	17	1.12	–	–	1.93	1.43	–	–	1.64
ALP (mU/mL)	0	284	244	253	248	244	263	294	241
	6	218	216	262	241	202	308*	395**	269
	13	168	168	260*	247	174	279*	674*	291
	17	99	–	–	187	140	–	–	200
Liver weight									
Absolute (g)	13	257	299	338	337*	258	247	323	340
Relative (%)									
To body	13	2.6	3.0	3.7**	3.7**	2.9	2.9	3.8*	3.8**
To brain	13	3.4	4.1	4.2	4.6**	3.4	3.4	4.3	4.4
Absolute (g)	17	241	–	–	341	254	–	–	319
Relative (%)									
To body	17	2.6	–	–	3.2	2.8	–	–	3.3
To brain	17	3.4	–	–	4.4	3.5	–	–	4.5

ALP: alkaline phosphatase; eq.: equivalents; ppm: parts per million; U: units; week 0: pretest value; –: no measurements; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$

[a] Because of the number of animals killed at week 17, no statistical analysis was performed for the recovery period.

Source: Briffaux (1989b)

and 0, 3.64, 78.3 and 1977 mg/kg bw per day for females, respectively). Clinical observations and feed consumption were recorded daily. Body weight was recorded weekly. A detailed veterinary examination and ophthalmoscopy were carried out at intervals during the study, and a range of haematological and clinical biochemistry (blood and urine) parameters was measured throughout the study. Lufenuron levels in the blood were analysed from all surviving dogs in weeks 34, 37 and 52. At the end of the scheduled period, the animals were killed and examined postmortem. Terminal blood and bone marrow samples were taken,

selected organs were weighed and specified tissues were taken for subsequent histopathology examination. In order to support the interpretation of the results obtained in this study and to demonstrate saturation, the concentrations of the test article were determined in blood, fat and brain samples. Blood samples were taken at 34, 37 and 52 weeks of feeding. At final kill after 24 months, fat and brain tissues were sampled from all surviving animals.

One mid-dose male died during week 33, and one male and one female were euthanized moribund during week 37. Neuromuscular signs were noted for these three animals. This generally consisted of tremors, salivation, dyspnoea, unsteady gait and convulsions. There were no other mortalities. Neuromuscular clinical signs were noted in 3/4 animals of each group fed with 2000 and 50 000 ppm after at least 20 weeks of treatment. These signs generally included stiffness, convulsions (tonic or clonic) and salivation, followed by tremors, decreased activity and unsteady gait. The incidence of these signs was biologically similar at 2000 and 50 000 ppm. At 2000 ppm, three animals died, but in dogs fed 50 000 ppm, neuromuscular signs were transient and reversible. A very high incidence of vomiting was noted throughout the treatment period for one female at 50 000 ppm. The ophthalmological examination did not reveal any treatment-related changes. A reduction in mean body-weight gain was seen in the 50 000 ppm males and females. At 2000 ppm, a slight decrease in body weight and a reduced body-weight gain were seen, particularly in the males. Feed consumption was not affected.

An increase in platelet count was noted in groups fed 2000 and 50 000 ppm. In comparison with pretest and control values, blood chemistry analyses showed a dose-related increase in cholesterol at several time points at 2000 and 50 000 ppm. Phospholipids were elevated in males at 2000 and 50 000 ppm and in females at 50 000 ppm. A dose–response relationship was evident in the males. ALP activity was higher in males and females at 2000 ppm and in males at 50 000 ppm. No statistically significant changes in ALAT activity were noted. However, in comparison with control animals, elevated ALAT values were noted in males and females at 2000 ppm and in males at 50 000 ppm. For one female at 2000 ppm, the elevated value noted at week 13 was associated with an increase in γ-glutamyltranspeptidase (GGT) activity. These elevated ALAT and GGT values were considered to be treatment related (Table 13).

Increases in mean adrenal weights (absolute and relative to body and brain weights) were noted in males and females at 2000 and 50 000 ppm. Increases in mean thyroid weights (absolute and relative to body and brain weights) were noted in males of all dose groups and in females at 2000 and 50 000 ppm. Increases in liver weights (absolute and relative to body and brain weights) were noted in males from all treated groups (not dose related) and in females at 2000 and 50 000 ppm (not dose related). This increase was statistically significant in

Table 13

Haematology, clinical biochemistry and organ weights in a 52-week oral study in dogs

	Males				Females			
Weeks	**0 ppm**	**100 ppm**	**2 000 ppm**	**50 000 ppm**	**0 ppm**	**100 ppm**	**2 000 ppm**	**50 000 ppm**
Platelet count	75	77	72	71	70	71	72	70
13	353	360	397	598**	325	356	383	421
26	317	343	448	629***	371	351	420	524
39	279	352	451*	622***	317	354	417	532**
52	289	363	532**	643***	346	345	469	572**
Cholesterol (g/L)	4.6	4.9	4.6	4.7	4.8	5.1	4.7	4.8
13	1.27	1.53	1.71	2.21*	1.22	1.16	1.72	1.83
26	1.24	1.55	1.60	2.09*	1.32	1.49	1.59	1.92
39	1.34	1.61	1.85	1.91	1.27	1.21	1.45	1.96
52	1.23	1.49	1.73	2.24*	1.53	1.34	1.68	2.17*
Phospholipids (g/L)	1.20	1.42	1.86*	1.98**	1.40	1.43	2.42*	2.09*
13	2.59	2.85	2.91	3.68*	2.42	2.36	3.00	3.26
26	2.66	2.99	3.04	3.45	2.71	3.08	2.90	3.48
39	2.74	2.78	3.16	3.36	2.33	2.48	2.64	3.45*
52	2.97	3.15	3.53	3.94	3.34	3.11	3.50	4.13
ALP (IU/L)	4.6	4.9	4.6	4.7	4.8	5.1	4.7	4.8
13	197	242	178	247	240	170	272	251
26	131	206	150	227	154	130	478	208
39	116	170	207	235	153	124	347	222
52	110	169	173	281*	154	151	323	254
ALAT (IU/L)	1.20	1.42	1.86*	1.98**	1.40	1.43	2.42*	2.09*
13	40	42	36	48	48	38	67	34
26	44	40	35	38	43	33	54	35
39	40	38	35	35	41	35	44	33
52	43	42	40	33	37	37	63	33
Adrenal weight								
Absolute (g)	1.349	1.270	1.606	1.844*	1.462	1.412	2.686**	1.562
Relative (%)								
To body	0.017 6	0.016 8	0.022 1	0.023 7*	0.019 7	0.020 4	0.036 7***	0.022 2
To brain	0.012 7	0.013 0	0.016 9	0.019 6*	0.016 6	0.015 8	0.031 9***	0.020 1
Thyroid weight								
Absolute (g)	0.652	0.970	0.834	0.944	0.549	0.618	0.761	0.791
Relative (%)								
To body	0.008 4	0.012 7	0.011 3	0.012 2	0.007 4	0.008 9	0.010 5	0.011 2
To brain	0.006 0	0.010 0	0.008 7	0.009 7	0.006 3	0.007 0	0.009 1	0.010 1
Liver weight								
Absolute (g)	261.7	331.8	432.7	387.2	262.5	281.0	419.3**	339.6

Table 13 (continued)

	Males				Females			
Weeks	0 ppm	100 ppm	2 000 ppm	50 000 ppm	0 ppm	100 ppm	2 000 ppm	50 000 ppm
Relative (%)								
To body	3.41	4.34	5.94**	4.96*	3.57	4.05	5.75***	4.81**
To brain	2.42	3.42	4.54**	4.03**	3.01	3.13	4.96***	4.33***

ALAT: alanine aminotransferase; ALP: alkaline phosphatase; IU: International Units; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$
Source: Briffaux (1992)

males and females at 2000 and 50 000 ppm for the relative values and in females at 2000 ppm for the absolute values (Table 13).

At the end of the treatment period, enlarged adrenals were noted in one female at 2000 ppm and pale area(s) in the lungs were observed in two animals at 2000 ppm and in 50 000 ppm animals. Histopathological lesions that could be attributed to treatment were seen in the liver, thyroid, adrenals and lungs. Hepatocytic hypertrophy was present in dogs fed 2000 and 50 000 ppm, associated with an increased intracellular dark pigmentation of the hepatocytes. Cell necrosis was noted in one female fed 2000 ppm. In the thyroid, follicular dilatation with increased eosinophilic staining of the colloid was seen in one dog at 100 ppm (grading: minimal), three dogs at 2000 ppm (grading: minimal [two animals] to slight [one animal]) and five dogs at 50 000 ppm (grading: minimal [one animal] to slight [four animals]). Cortical hyperplasia of the adrenal was present in dogs fed 2000 and 50 000 ppm, the highest severity being noted at 2000 ppm. In the lungs, intra-alveolar histiocytosis was seen generally associated with cholesterol clefts at 2000 and 50 000 ppm.

The blood, fat and brain tissue concentrations of lufenuron were similar at 2000 and 50 000 ppm. Thus, the blood and tissues seemed to be saturated to a similar extent at both of these feeding levels. At the low feeding level of 100 ppm, the blood and tissue concentrations were lower by a factor of approximately 10. The similar blood and tissue levels at 2000 and 50 000 ppm were in good agreement with the absence of a dose–response relationship for the majority of treatment-related findings. Major clinical signs (neuromuscular signs, convulsions) were seen after at least 20 weeks of treatment in three animals at 2000 ppm and in three animals at 50 000 ppm. Although the incidence was similar in both groups, the animals at 2000 ppm were more severely affected. At 50 000 ppm, neuromuscular signs were transient and reversible. In view of the small number of animals and the similar blood and tissue levels, the mortality at 2000 ppm and not at 50 000 ppm could have occurred by chance (Table 14).

Table 14
Blood, fat and brain concentrations in a 52-week oral toxicity study in dogs

Tissue	Week	Sex	0 ppm	100 ppm	2 000 ppm	50 000 ppm
Blood (µg/mL blood)	34	Males	<0.1	5.8	31.7	29.2
		Females	<0.1	4.3	29.7	26.7
	37	Males	<0.1	5.6	33.3	33.7
		Females	<0.1	4.7	29.2	30.7
	52	Males	<0.1	5.1	42.5	55.5
		Females	<0.1	6.3	34.7	41.7
Fat (mg/kg tissue)	52	Males	10.5[a]	670	6 000	5 025
		Females	9.2[a]	797	4 433	4 925
Brain (mg/kg tissue)	52	Males	<2	15.5	250	242.5
		Females	<2	9	282.5	197.5

ppm: parts per million
[a] Most probably due to contamination of samples.
Source: Briffaux (1992)

At 100 ppm, a slight increase in liver weight without any associated histopathological changes was observed, and one dog showed a slight increase in thyroid weight and histopathological evidence of minimal dilatation of the follicles. Considering the nature and low severity of these changes and that the histopathological changes in the thyroid were seen in only one animal, the NOAEL was 100 ppm (equal to 3.97 mg/kg bw per day for males and 3.64 mg/kg bw per day for females), based on mortality, neuromuscular signs, including convulsions, reduced body-weight gains and changes in clinical pathology parameters, organ weights and histopathological lesions in adrenals, liver, thyroid and lungs observed at 2000 ppm (equal to 65.4 mg/kg bw per day for males and 78.3 mg/kg bw per day for females) (Briffaux, 1992).

In another 1-year toxicity study, groups of four male and four female beagle dogs received lufenuron (purity 97.1%) in their diet at a concentration of 0, 10, 50, 250 or 1000 ppm (equal to 0, 0.31, 1.42, 7.02 and 29.8 mg/kg bw per day for males and 0, 0.33, 1.55, 7.72 and 31.8 mg/kg bw per day for females, respectively). Clinical observations and feed consumption were recorded daily. Body weight was recorded weekly. A detailed neurological examination and ophthalmoscopy were carried out at intervals during the study, and a range of haematological and clinical biochemistry (blood and urine) parameters was measured throughout the study. At the end of the scheduled period, the animals were killed and examined postmortem. Terminal blood and bone marrow samples were taken, selected organs were weighed and specified tissues were taken for subsequent histopathological examination. In order to support the interpretation of the results obtained in this study and to demonstrate saturation, the concentrations

of the test article were determined in blood, fat and brain samples. Blood samples were taken at 26 and 52 weeks of feeding. At final kill after 24 months, fat and brain tissues were sampled from all surviving animals.

One female at 1000 ppm was found dead in week 31. Prior to death, this animal had shown marked clinical signs and deterioration of general state of health. One female and two males from this dose group were euthanized in weeks 28, 43 and 49 following clinical signs of convulsions. Treatment-related clinical signs were restricted to 1000 ppm animals that died or were killed prior to scheduled termination. These signs included convulsions, tremor, atactic gait, reduced locomotor activity, aggressiveness, nervousness, impeded respiration, vomiting and salivation. During the first third of the treatment period, body-weight gain of treated animals was comparable with that of controls. After this time, body-weight loss was recorded in all 1000 ppm animals, resulting in an overall weight loss or depressed body-weight gain. At the end of the treatment period, one male at 250 ppm lost weight from week 47 onwards (~5%: 10.4 kg in week 52 compared with ~11.0 kg during weeks 14–46), whereas the mean body weight of males at 250 ppm in week 52 (12.00 kg) was comparable with the control value (12.08 kg). The results of the ophthalmoscopic examinations and measurement of neurological parameters showed no treatment-related effects.

Mean feed consumption for the treated groups was comparable with that of controls except for single periods of depressed feed intake at 1000 ppm caused by animals with clinical signs prior to early kill. There were no statistically significant differences. Males and females at 1000 ppm had minimally but consistently higher platelet counts throughout the treatment period. Alterations of blood chemistry parameters were confined to the 1000 ppm animals. Higher activities of ALP were recorded in both sexes at weeks 26 and 52, and males also had higher GGT activities at week 52. Plasma globulin levels were slightly increased in both sexes at the end of treatment and in males at week 26. Higher plasma cholesterol levels were observed in males at weeks 13, 26 and 52 and in females at week 52. Also, males had minimally lower plasma phosphate and thyroxine levels, whereas females showed higher plasma glucose levels at each assessment period during treatment (Table 15).

There was a dose-related increase in both absolute and relative (to body weight) liver weights in both sexes at 250 ppm (absolute: 126% of control values for both sexes; relative: 129% and 135% of control values for males and females, respectively) and in the surviving animals at 1000 ppm (absolute: 147% and 141% of control values for males and females, respectively; relative: 164% and 172% of control values for males and females, respectively). Mean absolute and relative adrenal weights were increased in the surviving animals at 1000 ppm. In addition, a tendency to an increase in adrenal weights was recorded in one male

Table 15

Haematological and blood chemistry findings in a 52-week oral toxicity study in dogs

	Males					Females				
Parameter / week	**0 ppm**	**10 ppm**	**50 ppm**	**250 ppm**	**1 000 ppm**	**0 ppm**	**10 ppm**	**50 ppm**	**250 ppm**	**1 000 ppm**
Platelets (g/L)										
–1	264.8	287.8	295.0	268.5	249.8	254.3	267.8	284.0	247.0	298.8
13	248.5	294.0	299.0	259.0	314.0	272.0	292.8	283.0	301.3	364.8*
26	243.0	354.8*	327.0*	337.8*	350.6*	294.8	322.3	308.0	299.8	454.5
52	274.5	381.3	311.5	386.3	405.0	305.3	307.8	356.0	383.0	654.0+
ALP (U/L)										
–1	122.3	85.45	88.28	112.5	105.6	84.05	72.80	93.53	96.63	74.68
13	97.48	68.40	73.08	83.70	92.65	68.60	57.53	81.15	76.16	74.48
26	67.55	47.88	54.45	58.70	135.6*	48.15	46.50	64.33	70.18	113.9*+
52	53.30	40.53	46.50	59.23	144.1	48.03	47.90	65.78	67.68	173.9
GGT (U/L)										
–1	1.375	2.000	1.375	3.375*	0.725	1.375	2.200	0.550	2.950	2.925
13	4.450	4.325	4.625	3.500	4.800	4.175	3.800	4.300	4.175	4.625
26	4.700	4.150	4.050	3.625	4.550	4.150	3.225	3.625	4.313	5.338
52	2.850	2.700	3.325	3.025	5.100	1.750	3.350	3.350	3.888	3.650
Globulin (g/L)										
–1	21.01	22.72	21.34	21.28	22.71	21.18	20.76	21.60	19.45	20.50
13	24.02	25.10	23.05	23.86	24.47	22.70	23.34	22.21	21.87	22.17
26	24.44	25.23	23.99	24.22	27.02	22.38	23.57	22.62	21.83	22.36
52	25.89	28.09	25.14	26.95	30.61	23.88	25.95	24.99	24.25	27.40
Cholesterol (mmol/L)										
–1	3.475	3.408	3.630	3.593	4.061*	3.305	3.170	3.135	3.203	3.245
13	3.868	3.720	3.873	3.885	4.985*	3.513	3.708	3.588	3.515	3.805
26	3.840	3.113*	3.855	3.998	5.063*+	3.320	3.735	3.558	3.413	3.863
52	3.610	3.193	3.688	3.875	4.735	3.458	3.450	3.465	3.460	4.465
Phosphate (mmol/L)										
–1	2.138	2.010	2.225	2.035	2.015	1.813	1.945	1.890	1.973	1.920
13	1.628	1.533	1.675	1.588	1.350*	1.385	1.340	1.345	1.398	1.350
26	1.468	1.378	1.458	1.230*	1.114*–	1.306	1.298	1.298	1.124	1.045
52	1.185	1.275	1.175	1.083	0.810	1.063	1.115	0.858	0.985	0.828
Thyroxine (nmol/L)										
–1	37.84	36.73	31.25	33.32	35.00	33.48	35.06	36.10	32.35	41.65
13	35.20	32.26	28.40	32.40	27.03	33.50	35.15	35.27	32.54	31.02
26	33.75	34.37	27.00	25.17*–	25.42–	25.02	28.73	33.50	27.38	30.21
52	43.71	40.39	37.19	34.99	29.43	43.02	40.88	45.34	35.93	33.57
Glucose (mmol/L)										
–1	5.715	6.110	5.840	5.818	6.108	5.900	6.023	5.723	5.770	6.270
13	5.143	5.420	5.430	5.385	6.008*–	5.065	5.455	5.213	5.365	6.050*

Table 15 (continued)

Parameter / week	Males					Females				
	0 ppm	10 ppm	50 ppm	250 ppm	1 000 ppm	0 ppm	10 ppm	50 ppm	250 ppm	1 000 ppm
26	5.420	5.323	5.495	5.350	6.185	5.275	5.953	5.560	5.665	8.820*
52	5.120	5.035	5.145	5.203	6.200	5.235	5.558	5.490	5.278	6.480

ALP: alkaline phosphatase; GGT: γ -glutamyltranspeptidase; ppm: parts per million; U: units; *: $P < 0.05$ (Wilcoxon); +/–: $P < 0.01$ (Jonckheere trend test)
Source: Altmann (1995)

and one female at 250 ppm, but without pathological correlates. Mean thymus weight was slightly decreased in the surviving males and females at 1000 ppm.

At necropsy, the liver was described as enlarged in one dog of each sex at 1000 ppm, and enlarged adrenal glands were found in most animals in this group. Microscopically, a minimal to moderate hypertrophy of hepatocytes was found in males and females at 250 and 1000 ppm. Pigmentation of liver Kupffer cells was increased in incidence and severity in the 1000 ppm males. A minimal to marked diffuse adrenal cortical hyperplasia was seen in all animals at 1000 ppm. In a few females in this group, cortical haemorrhage was also present. A minimal to moderate depletion of lymphocytes of the Peyer's patches showed an increased incidence in the small intestine of the 1000 ppm animals. Depletion of lymphocytes in the mesenteric lymph node was increased in incidence and severity in both 1000 ppm groups. Moderate to marked thymic atrophy showed an increased incidence and severity in 1000 ppm females. Aggregation of alveolar foam cells was found in the lungs of both sexes at 1000 ppm. Other findings noted at microscopic examination were considered to be unrelated to treatment. No effects were observed on thyroid weight or histopathology (Table 16).

The blood, fat and brain concentrations were similar in males and females. The blood levels at week 52 were similar to or only slightly higher than those at week 26, indicating that a plateau was reached. The blood-to-fat ratio was approximately 1:100 or slightly higher. In the brain, the concentrations were similar to those in the blood at the two lowest feeding levels and up to about 5 times higher than those in the blood at the two highest dose levels (Table 17).

The effects observed at a dose level of 250 ppm were confined to body-weight loss in one male, a tendency to increased adrenal weight in one male and one female, without pathological correlates, and increased absolute and relative liver weights in males and females with hypertrophy of hepatocytes. The liver effect should be seen as an adaptive response, in the absence of further histopathological damage and relevant clinical chemistry changes. The effects on body weight and adrenals at this dose had no effect on the group means.

Table 16
Pathology findings in a 52-week oral toxicity study in dogs

	Males ($n = 4$)					Females ($n = 4$)				
Parameter	**0 ppm**	**10 ppm**	**50 ppm**	**250 ppm**	**1 000 ppm**	**0 ppm**	**10 ppm**	**50 ppm**	**250 ppm**	**1 000 ppm**
Mortality (weeks)	0	0	0	0	2 (43/49)	0	0	0	0	2 (28/31)
Body weight (kg)	11.56	10.88	11.10	11.44	10.53	10.64	10.14	10.42	9.905	8.740
Liver										
Liver weight										
Absolute (g)	356.3	359.6	354.6	451.8	523.0*	326.1	299.4	334.3	410.6*	462.1
Relative (%)	30.72	33.08	32.01	39.84	50.45	30.77	29.67	32.07	41.43*	52.95+
Enlarged liver	0	0	0	0	1	0	0	0	0	1
HH	0	0	0	4	3	0	0	0	3	1
KCP	0	1	1	1	2	0	0	0	0	0
Adrenals (both)										
Adrenal weight										
Absolute (g)	1.470	1.461	1.583	1.732	2.485+	1.606	1.728	1.613	1.819	3.078
Relative (%)	0.127	0.134	0.143	0.154	0.236+	0.151	0.173	0.155	0.184	0.356
Enlarged adrenals	0	0	0	0	3	0	0	0	0	4
ACH	0	0	0	0	4	0	0	0	0	4
Thymus										
Thymus weight										
Absolute (g)	6.714	4.899	7.549	7.410	4.673	8.429	7.611	6.101	6.051	3.055
Atrophy	0	0	0	0	0	2	2	3	3	4
Thyroid										
Thyroid weight										
Absolute (g)	1.162	1.088	1.034	1.077	1.079	0.820	1.040	0.960	0.981	1.020
Relative (%)	0.010 1	0.010 0	0.009 3	0.009 4	0.010 7	0.007 7	0.010 7	0.009 2	0.009 9	0.011 8
Small intestine										
HPP	0	0	0	0	4	0	0	0	0	3
Mesenteric lymph node										
PC	4	3	4	4	4	0	0	0	0	0
HLT	0	1	0	0	3	1	1	0	1	3
Lungs										
AFC	0	0	0	0	3	0	0	0	0	1

ACH: adrenocortical hyperplasia; AFC: alveolar foam cells; HH: hypertrophy of hepatocytes; HLT: hypocellularity of lymphatic tissue; HPP: hypocellularity of Peyer's patch; KCP: Kupffer cell pigmentation; PC: phagocytic cells; ppm: parts per million; *: $P < 0.05$; +: $P < 0.01$
Source: Altmann (1995)

Table 17
Blood, fat and brain concentrations in a 52-week oral toxicity study in dogs

Tissue	Week	0 ppm	10 ppm	50 ppm	250 ppm	1 000 ppm
Blood (µg/mL blood)	26	<0.1	0.19	1.7	10	41
	52	<0.1	0.18	1.9	24	66
Fat (mg/kg tissue)	52	<0.5	28	260	2 500	8 700
Brain (mg/kg tissue)	52	<0.1	0.2	1.9	62	340

ppm: parts per million
Source: Altmann (1995)

The NOAEL was 250 ppm (equal to 7.02 mg/kg bw per day for males and 7.72 mg/kg bw per day for females), based on treatment-related mortality and clinical findings, effects on body weight and effects on the liver and adrenals, with associated histopathology and/or clinical chemistry changes, at 1000 ppm (equal to 29.8 mg/kg bw per day for males and 31.8 mg/kg bw per day for females) (Altmann, 1995).

(ii) Metabolite CGA 224443

Rats

In a 28-day oral toxicity study, CGA 224443 (93.2% purity) was admixed in the diet at target concentrations of 0, 100, 500, 2000 or 8000 ppm and fed ad libitum to Tif: RAIf (SPF) Sprague Dawley–derived albino rats (5 or 10/sex per group). Following the dosing period, control and high-dose rats (5/sex per group) were maintained on plain diet for a further 4 weeks to study the reversibility of effects. The mean analytically verified intakes of CGA 224443 were 8.8, 30, 118 and 437 mg/kg bw per day for males and 9.0, 31, 112 and 360 mg/kg bw per day for females, respectively. During the first 4 days of treatment, the control animals were inadvertently given feed contaminated with the test compound at approximately 40 ppm. Uncontaminated diet was provided from day 5 onwards. Deaths, clinical signs, body weight and feed consumption were recorded throughout the dosing period. Standard laboratory investigations (haematology, clinical chemistry and urine analysis) were performed on blood and urine samples obtained at termination of the dosing and recovery periods. Following scheduled kill, all animals underwent gross necropsy and organs were weighed and spleen, heart, liver, kidneys, adrenal glands and organs with gross lesions were histopathologically examined.

There were no treatment-related deaths, clinical signs or toxicologically significant effects on absolute and relative organ weights. In males and females, feed consumption during the first week of dosing was depressed by 56% and 70% ($P < 0.05$) at 8000 ppm and by 13% and 22% (statistically insignificant) at 2000

ppm. At the high dose, intake remained depressed by up to 13% in males and 38% in females throughout the following 3 weeks; although normalizing in males during recovery, intake remained up to 16% below control values in females. Mean body weight was decreased by up to 15% and 21% in males and females at 8000 ppm throughout the dosing period ($P < 0.05$) and by 8% in females at 2000 ppm during week 2. After the recovery period, body weight remained depressed by 6% in males and 13% in females at 8000 ppm.

No treatment-related effects on urine analysis parameters were observed. Selected haematological, clinical chemistry, organ weight and histopathological findings are summarized in Table 18. Rats treated at and above 500 ppm were dose-relatedly affected by normochromatic regenerative anaemia, characterized by statistically significant ($P < 0.05$) depression of erythrocyte count, haematocrit and blood haemoglobin concentration, up to 40% elevation in blood bilirubin level, increased methaemoglobin formation at 2000 and 8000 ppm, and reticulocytosis at the high dose. Minimal to moderate splenic congestion, haemosiderosis and extramedullary haematopoiesis were found in some females and/or males at 500 ppm and both sexes at 2000 and 8000 ppm. The splenic haemosiderosis was more severe in females than males, but persisted in both sexes after the recovery period. The remaining haematological and histopathological features were reversible.

Several indices of hepatocellular function were perturbed reversibly at the upper doses. Significant ($P < 0.05$) dose-related increases of up to 24% in blood aspartate transaminase activity occurred in males at 2000 and 8000 ppm. At 8000 ppm only, blood cholesterol level and ALP and GGT activities increased in both sexes, and alanine transaminase activity was elevated by 32% in males but depressed in females ($P < 0.05$). In the centrilobular and midzonal regions of the liver, minimal to moderate hepatocytic hypertrophy (a possible indicator of metabolic enzyme induction) was observed in most or all rats treated at 2000 and 8000 ppm. Correlating with the anaemia were minimal hepatic extramedullary haematopoiesis (which occurred in a male at 500 ppm and both sexes at 2000 and 8000 ppm) and minimal to moderate haemosiderin pigmentation of Kupffer cells (observed in a female at 2000 ppm and both sexes at the high dose). These histopathological features were reversible.

The NOAEL was 100 ppm (equal to 8.8 mg/kg bw per day), based on the development of anaemia and hepatic extramedullary haematopoiesis at and above a dietary concentration of 500 ppm (equal to 30 mg/kg bw per day) (Gerspach, 1993).

Table 18
Effects of exposure to CGA 224443 in a 28-day dietary toxicity study in rats [a]

	Males					Females				
Parameter	0 ppm	100 ppm	500 ppm	2 000 ppm	8 000 ppm	0 ppm	100 ppm	500 ppm	2 000 ppm	8 000 ppm
Haematology										
RBC (T/L)	7.946	7.830	7.595*	↓7.134*	↓6.466*	7.709	7.746	7.131	↓6.812*	↓6.249*
Hb (mmol/L)	9.640	9.460	↓ 9.100*	↓8.740*	↓8.105*	9.320	9.300	8.720	↓8.420*	↓7.875*
Haematocrit (L)	0.461	0.456	0.449	↓0.433*	↓0.410*	0.442	0.450	0.422	0.415*	↓0.404*
MCV (fL)	58.05	58.28	59.09	60.68*	↑63.41*	57.38	58.18	59.41	↑60.86*	↑64.64*
MCHC (mmol/L)	20.88	20.78	20.30*	↓20.24*	↓19.81*	21.05	20.65	20.68	↓20.33*	↓19.49*
MetHb (L)	0.007	0.007	0.007	↑0.009*	↑0.009*	0.006	0.007	0.007	↑0.011*	↑0.010*
Reticulocytes (L)	0.022	ND	ND	ND	↑0.075*	0.018	ND	ND	ND	↑0.093*
Clinical chemistry										
Total bilirubin (µmol/L)	1.768	1.802*	2.182	2.226*	↑2.513*	2.227	2.276	2.464*	↑2.892*	↑2.821*
Cholesterol (mmol/L)	1.706	1.518	1.628	1.730	↑2.339*	1.851	1.774	1.681	2.248	↑2.581*
ALP (U/L)	123.0	116.1	122.3	115.1	129.7	80.90	86.86	69.42	68.48	101.1*
ALT (U/L)	24.03	22.28	22.10	22.90	31.76*	20.17	19.34	19.52	17.58	↓15.71*
AST (U/L)	56.32	62.10	61.96	↑69.70*	↑69.34*	58.66	57.40	62.86	56.50	56.96
GGT (U/L)	0.210	0.000	0.000	0.000	0.270	0.000	0.000	0.000	0.000	0.270
Liver [a]										
Extramedullary haematopoiesis	0	0	1	3	3	0	0	0	4	3
Kupffer cell pigmentation	0	0	0	0	2	0	0	0	1	4
Hepatocytic hypertrophy	0	0	0	5	5	0	0	0	4	5
Congestion	0	0	4	5	5	0	0	0	5	5
Spleen										
Haemosiderosis	0	0	1	3	5	0	0	3	5	5
Extramedullary haematopoiesis	0	0	3	5	5	0	0	0	4	3

ALP: alkaline phosphatase; ALT: alanine transaminase; AST: aspartate transaminase; GGT: γ -glutamyltranspeptidase; Hb: haemoglobin; MCHC: mean cell haemoglobin concentration; MCV: mean cell volume; MetHb: methaemoglobin; RBC; red blood cells; *: $P < 0.05$

[a] Results expressed as no. of rats with the finding. There were 10 rats/sex per group at 0 and 8000 ppm; 5 rats/sex per group at 100, 500 and 2000 ppm.

Source: Gerspach (1993)

(b) Dermal application

Rats

In a 28-day dermal toxicity study, groups of five male and five female albino rats (Tif: RAIf (SPF), hybrids of RII/1 × RII/2) were treated dermally with lufenuron (purity 95.2%) moistened with 0.5% carboxymethylcellulose in 0.1% aqueous polysorbate 80 (wetting agent) at a dose level of 0, 100, 300 or 1000 mg/kg bw

per day, 5 days/week, for 4 weeks. The exposure period was 6 hours/day under an occlusive dressing. Mortality and signs of systemic toxicity were determined daily, and signs of local skin irritation were determined approximately 17 hours after removing the gauze patches. Body weights and feed consumption were recorded weekly. Laboratory investigations (haematology and blood chemistry) were carried out on all surviving animals of each dose group at the end of the treatment period. At the end of the test period, all control and treated rats underwent detailed necropsy. Besides the weight of the exsanguinated body, the following organs were weighed: brain, heart, liver, kidneys, adrenals, thymus, ovaries/testes and spleen. Selected organs and tissues were preserved. The following samples were microscopically examined: skin application site, skin remote site, brain, liver, kidneys, adrenal glands, thymus and spleen (histopathological evaluation of paired organs was performed on both of them).

There were no systemic or local effects at any dose level. The NOAEL was 1000 mg/kg bw per day, the highest dose tested (Schneider, 1990).

(c) Exposure by inhalation

No study was submitted.

2.2.3 Long-term studies of toxicity and carcinogenicity

Mice

In a chronic toxicity and carcinogenicity study, groups of 60 male and 60 female mice (Tif: MAGf (SPF), hybrids of NIH × MAG) received lufenuron (purity 96.2%) in their diets at a concentration of 0, 2, 20, 200 or 400 ppm (equal to 0, 0.222, 2.25, 22.6 and 62.9 mg/kg bw per day for males and 0, 0.217, 2.12, 22.0 and 61.2 mg/kg bw per day for females, respectively) for 18 consecutive months. As a result of high mortality in the high-dose group, surviving animals in this dose group were terminated in weeks 9 and 10. Samples were taken from selected animals at weeks 53 and 78 for haematology. Clinical chemistry parameters were not assessed. At termination, all surviving animals underwent a gross necropsy, and selected organs were weighed. Gross necropsies were performed for all animals that died prior to scheduled termination (found dead or euthanized moribund). Samples of selected tissues were processed for histopathological evaluation from all surviving test animals and from any unscheduled deaths that occurred during the study.

During the first 9 weeks of the study, five males and 29 females at 400 ppm were found dead. All survivors in this group were killed early in weeks 9 and 10. The mortality recorded at 2 and 20 ppm was similar to control values, but the mortality rate at 200 ppm was higher than for controls. The percentage of animals surviving to termination at 18 months was 76%, 74%, 84% and 64% for the males

and 82%, 82%, 88% and 48% for the females at 0 (controls), 2, 20 and 200 ppm, respectively. Tonic–clonic convulsions, occurring spontaneously or in response to external stimuli such as handling, were exhibited by a number of animals of both sexes at 200 and 400 ppm. The convulsions were of short duration (up to 30 seconds), during which time forelimb paddling, uncoordinated movement, jumping and straub tail occurred. Convulsions recorded in the other dose groups, including controls, were of a shorter duration and less intense (tonic phase only) and were regarded as spontaneous events, unrelated to treatment. No other clinical signs of reaction to treatment were observed.

No effects on body weight were recorded for either sex at 2 and 20 ppm or for males at 200 ppm. Females at 200 ppm showed a small, nonstatistically significant reduction in mean body weights compared with controls during the last 6 months of the study. Feed consumption for both sexes at 2, 20 and 200 ppm was not adversely affected by treatment. No treatment-related haematological changes were noted in either sex at either 12 months or study termination (18 months). Slight increases in absolute and relative (to body weight) adrenal weights were observed in the 200 ppm females compared with controls (absolute weight: 113%; relative weight: 122%). However, all values were within the range recorded among controls, and microscopy revealed no morphological changes in this organ. Macroscopic postmortem examination revealed a higher incidence of single nodules of the lung in male mice at 2 ppm and of single and multiple nodules of the lung in male mice at 200 ppm compared with controls. The incidence at 20 ppm was similar to that of controls, indicating no dose–response relationship.

Microscopic examination revealed higher incidences of fatty liver compared with controls, accompanied by necrotic changes in females at 200 ppm. Similar findings were seen at 400 ppm. Males at 200 ppm had a higher incidence of inflammatory changes in the prostate. Males in this group also showed an increased incidence, compared with controls, of both single and multiple lung adenomas, relating to the increased numbers of lung nodules seen macroscopically. The incidences observed across the treated groups in this study were not dose related, suggesting that the variation in incidence was spontaneous rather than as a result of treatment. In addition, the incidence of lung adenomas is known to be very variable in aged mice, and thus these isolated higher incidences were not considered to indicate an effect of treatment (Table 19).

The NOAEL for systemic toxicity was 20 ppm (equal to 2.25 mg/kg bw per day for males and 2.12 mg/kg bw per day for females), based on increased mortality, clinical signs (tonic–clonic convulsive episodes), increased incidences of fatty liver (in females accompanied by necrotic changes) and a higher incidence of inflammatory changes in the prostate at 200 ppm (equal to 22.6 mg/kg bw per day for males and 22.0 mg/kg bw per day for females).

Table 19
Pathology findings in an 18-month oral toxicity and carcinogenicity study in mice

	Incidence of finding									
	Males					Females				
Observation	**0 ppm**	**2 ppm**	**20 ppm**	**200 ppm**	**400 ppm**	**0 ppm**	**2 ppm**	**20 ppm**	**200 ppm**	**400 ppm**
Number examined	60	60	60	60	60	60	60	60	60	60
Lung										
Adenoma	12	19	4	19	0	8	5	8	7	0
Liver										
Fatty change	42	41	38	53	37	46	39	46	56	52
Recent necrosis	4	3	1	0	0	8	6	8	13	20
Hepatocellular necrosis	4	10	7	7	1	5	2	5	12	6
Prostate										
Total inflammatory lesions	3	6	4	10	1	–	–	–	–	–

ppm: parts per million
Source: Bachmann (1993a)

No treatment-related tumours were observed in male or female mice (Bachmann, 1993a).

Rats

In a combined chronic toxicity and carcinogenicity study in rats, groups of Tif: RAIf (SPF) rats (70/sex in main groups, 10/sex in satellite groups) received lufenuron (purity 96.2%) in their diets at a concentration of 0, 5, 50, 500 or 1500 ppm (equal to 0, 0.19, 1.93, 20.4 and 108 mg/kg bw per day for males and 0, 0.23, 2.34, 24.8 and 114 mg/kg bw per day for females, respectively) for 104 weeks. As a result of overt toxicity at 1500 ppm, all animals in this group were terminated in week 14. Clinical observations (including ophthalmology), body weights and feed and water consumption were measured throughout the study. Haematology, blood chemistry and urine analysis were carried out on selected animals periodically throughout the study and at the end of the treatment period. At the end of the scheduled period, all animals were killed and examined postmortem, selected organs were weighed and specified tissues were taken for subsequent histopathology. Samples of fat and blood were taken for analytical investigation from all animals killed at 1 year and from selected surviving animals at study termination; the results were reported as a separate study that was not submitted.

As a result of overt toxicity recorded at 1500 ppm, all animals in this group were prematurely killed in week 14. The mortality rate in the remaining treated groups was similar to that of the controls. The percentage of animals surviving to scheduled kill at 18 months was 50%, 46%, 57% and 59% for the males and 51%, 57%, 59% and 61% for the females at 0 (controls), 5, 50 and 500 ppm, respectively. A total of 46 males and 57 females at 1500 ppm and 47 males and 58 females at 500 ppm exhibited whole-body tonic–clonic convulsions from weeks 6 to 7. The convulsive episodes, lasting approximately 30–90 seconds, were observed mainly during and after handling and occurred repeatedly over several weeks in the majority of affected animals. As the study progressed, the incidence of convulsions at 500 ppm diminished. A low frequency of spontaneous convulsive episodes was seen in the control and low-dose groups. Several females at 500 ppm had vaginal discharge during the latter part of the treatment period.

Males and females at 1500 ppm had body weights approximately 10% lower than those of controls in week 12 prior to premature kill. Slightly lower mean body weights were recorded for both sexes at 500 ppm up to week 27. Thereafter, an increased body-weight gain in the females resulted in mean body weights approximately 20% higher than control values by the end of the study. The males at 500 ppm continued to have body weights slightly lower than control values to the end of the study (92% of controls). Higher feed intake, compared with controls, was recorded in week 1 for both sexes at 500 and 1500 ppm (111–114% of controls). Thereafter, the feed intake at 1500 ppm tended to be lower than that of controls up to the early termination of this group. Lower feed intake, compared with controls, was recorded in males at 500 ppm for the next 26 weeks (95% of controls), after which time the feed intakes were similar to control values. Females at 500 ppm showed an 18% increase in feed intake from week 23 to termination.

The week 13 examination of the blood, 1 week prior to the termination of the 1500 ppm animals, showed 8/20 males in this group with platelet counts above the concurrent control range and 3/20 females with white blood cell counts above the control range. Other parameters in this group were not affected by treatment, and subsequent examinations revealed no evidence of a treatment-related influence on the haematological profile of treated rats. Slightly lower levels of plasma protein and albumin and higher levels of plasma potassium and inorganic phosphorus were recorded for females at 1500 ppm prior to termination of this group in week 14. No clear effect of treatment on the blood chemistry profile of males or females was seen at 5, 50 or 500 ppm throughout the study. A number of differences between the mean values attained a level of statistical significance, but the differences were small and considered to be of no biological relevance. No effects on the urinary parameters were observed.

No treatment-related effects on organ weights were recorded in this study. Compared with the controls, statistical significance was achieved for absolute and relative adrenal gland weights for females at 50 and 500 ppm killed at week 53. However, no changes were detected in the adrenals by microscopy. Therefore, the difference at week 53 was considered to have occurred by chance.

At macroscopy, higher incidences of mottled lungs in males and females at 500 ppm were considered to be treatment related. Microscopic examination revealed an increased incidence of pulmonary alveolar foam cells in both sexes at 500 and 1500 ppm, with a higher severity level in the 500 ppm animals. This lesion was considered to be associated with a dilatation of the right heart ventricle developing due to increased pulmonary pressure caused by massive aggregations of foam cells in some females at 500 ppm.

Large adrenal glands were reported in 2/80, 5/80, 5/80 and 8/80 males and 3/80, 4/80, 2/80 and 11/80 females at 0, 5, 50 and 500 ppm, respectively. On microscopic examination, various lesions, such as cysts, sinusoidal cystic dilatations and hyperplasias or tumours of cortex or medulla, were found in these adrenals. The incidences of these lesions in the different groups did not indicate any treatment-related effect. The microscopic verification of some other macroscopic findings reported in higher numbers of treated animals revealed no treatment-related gross pathological effects.

An increased incidence of ulcerative and inflammatory lesions was seen in the non-glandular stomachs of males and females at 500 ppm. Focal haemorrhagic, necrotic, ulcerative and inflammatory lesions were found in the caecum and/or colon of both males and females at 500 and 1500 ppm. An increased incidence of fatty change was seen in the perilobular region of the liver in females at 500 ppm. In addition, inflammation of the female urinary tract was markedly increased at 500 ppm (Table 20).

No treatment-related increase in the incidence of hyperplastic or neoplastic lesions was found. The incidence of animals (n = 80) bearing primary tumours was 49, 52, 55, 42 and 0 for males and 57, 67, 54, 41 and 0 for females at 0, 5, 50, 500 and 1500 ppm, respectively. Evidence of an apparently statistically significant dose-related effect at 500 ppm was shown for the benign interstitial cell tumour of testis found at incidences of 2/80 at 0 ppm, 2/80 at 5 ppm, 1/80 at 50 ppm and 5/80 (6.25%) at 500 ppm, as well as the benign granular cell tumour of the cerebral meninges found in male animals at incidences of 1/80 at 0 ppm, 0/80 at 5 ppm, 1/80 at 50 ppm and 3/80 (3.75%) at 500 ppm. Both tumour incidences were in line with the historical control data presented in the study report, even though the historical control data were not completely within JMPR standards (i.e. rat strain not specified and presented studies were from 1978 to 1989, which is not within 2 years of the in-life phase of the lufenuron study). The historical control data from the laboratory and from the Registry of Industrial Toxicology

Table 20
Selected microscopic findings in a 2-year oral toxicity and carcinogenicity study in rats

	Incidence of finding									
	Males ($n = 80$)					Females ($n = 80$)				
Observation	0 ppm	5 ppm	50 ppm	500 ppm	1 500 ppm	0 ppm	5 ppm	50 ppm	500 ppm	1 500 ppm
Non-neoplastic findings										
Lung: foam cells										
Minimal	20	24	19	24	61	21	25	21	22	69
Moderate	12	12	10	19	6	14	15	16	24	5
Marked	5	4	5	11	0	8	3	6	19	0
Heart: dilatation of right ventricle	6	5	3	4	0	4	1	4	12	0
Non-glandular stomach: ulceration	0	0	1	4	0	1	0	1	2	0
Stomach: chronic inflammation	2	0	1	4	0	0	1	4	1	11
Caecum/colon: lesions total count	0	0	0	5	10	1	1	1	5	6
Liver (centrilobular): fatty change	0	1	0	0	0	2	2	1	36	0
Urinary bladder: inflammation	10	5	3	1	0	1	2	2	22	0
Kidney: inflammation of pelvis	11	5	2	2	0	2	4	2	20	0
Neoplastic findings										
Testis: benign interstitial cell tumour	2	2	1	5	–	–	–	–	–	–
Cerebral meninges: benign granular cell tumour	1	0	1	3	–	0	0	0	1	–

ppm: parts per million
Source: Bachmann (1993b)

Animal-data (RITA) database show a variable incidence, with no obvious change in incidence over the period 1978–1989. On this basis, it can be considered that all these data are applicable historical control data for the lufenuron 2-year rat study, which started in January 1990 (see Appendix 1).

Treatment with lufenuron for 24 months resulted in whole-body tonic–clonic convulsions at 500 and 1500 ppm. The extent of the reaction showed that the maximum tolerated dose was exceeded at the high dose level of 1500 ppm, and this group was prematurely killed in week 14. At 500 ppm, an initially reduced body-weight gain was observed, which in females reversed to a marked

increase in body weights from week 27 onwards, associated with a marked increase in feed intake. Histopathological changes at 500 and/or 1500 ppm included an increase in the incidence of pulmonary alveolar foam cells, ulcerative and inflammatory lesions in the non-glandular stomach and focal lesions in the caecum and/or colon. Additionally, increased incidences of fatty change in the liver and inflammation of the urinary tract were detected in females. There was no evidence of a tumorigenic response.

The NOAEL for systemic toxicity was 50 ppm (equal to 1.93 mg/kg bw per day for males and 2.34 mg/kg bw per day for females), based on tonic–clonic convulsions, decreased body weight and (histo)pathological effects on lungs, liver, non-glandular stomach, intestines and urinary tract at 500 ppm (equal to 20.4 mg/kg bw per day for males and 24.8 mg/kg bw per day for females). There was no evidence of a tumorigenic response (Bachmann, 1993b).

2.2.4 Genotoxicity

The results of studies of genotoxicity with lufenuron and the metabolites CGA 149772 and CGA 224443 are summarized in Table 21. All the studies were certified to comply with GLP and performed according to internationally accepted guidelines. No evidence of genotoxicity was observed.

2.2.5 Reproductive and developmental toxicity

(a) Multigeneration studies

In a two-generation study on reproductive toxicity, groups of 30 male and 30 female Sprague Dawley–derived Tif: RAIf (SPF) rats received lufenuron (purity 96.2%) in their diets at a concentration of 0, 5, 25, 100 or 250 ppm over the course of two generations (P and F_1). Mean lufenuron intakes for combined P and F_1 generations during the premating period were 0, 0.41, 2.1, 8.3 and 20.9 mg/kg bw per day for males and 0, 0.44, 2.2, 8.9 and 22.2 mg/kg bw per day for females, respectively.

After 10 weeks, the animals were mated (1:1) within each dose group and allowed to rear the ensuing F_1 litters to weaning. Litters were culled to four male and four female pups, where possible, on day 4 postpartum. The breeding programme was repeated with the F_1 parents selected from the F_1 offspring. Test diets were fed continuously throughout the study. Parental feed consumption and body weights were measured throughout the study. Reproductive performance, pup survival and developmental parameters were measured. Gross necropsy findings and histopathological observations in target organs of parental animals and pups not selected for mating were recorded.

There were no deaths or treatment-related clinical observations in either generation of parents. There was an increased incidence of skin wounds/crust on

Table 21
Results of studies of genotoxicity with lufenuron and metabolites

End-point	Test object	Concentration	Purity (%)	Result	Reference
Lufenuron					
In vitro					
Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535, T1537	20–5 000 µg/0.1 mL (±S9)	95.2	Negative	Deparade (1988a)
Mammalian cell gene mutation	V79 Chinese hamster cells, HPRT test	37.5–900 µg/mL (+S9) and up to 25–500 µg/mL (–S9)	95.2	Negative	Dollenmeier (1988)
Chromosomal aberration	CCL 61 CHO cells	200–1 600 µg/mL (+S9) and up to 25–200 µg/mL (–S9)	95.2	Negative	Strasser (1989)
Unscheduled DNA synthesis	Male Tif: RAIf (SPF) rat hepatocytes	2–2 000 µg/mL	95.2	Negative	Hertner (1988)
Unscheduled DNA synthesis	Human fibroblasts	28.40–6 900 µg/mL	95.2	Negative	Meyer (1988)
Unscheduled DNA synthesis	Cultured human MRC-9 lung cells	0.15–5 µg/mL (±S9)	97.1	Negative	Tanaka (1997)
In vivo					
Mouse micronucleus	Tif: MAGF, SPF mice	0, 1 250, 2 500 or 5 000 mg/kg bw (gavage)	95.2	Negative	Meyer (1989a)
Unscheduled DNA synthesis	Male Tif: RAIf (SPF) rats	0, 1 250, 2 500 or 5 000 mg/kg bw (gavage)	97.1	Negative	Hertner (1994)
Unscheduled DNA synthesis	Male HanIbM: WIST (SPF) rats	0, 1 000 or 2 000 mg/kg bw (gavage)	97.1	Negative	Ogorek (2000)
Unscheduled DNA synthesis	Male Tif: RAIf (SPF) rat hepatocytes	2–2 000 µg/mL	95.2	Negative	Hertner (1988)
Metabolite CGA 149772					
In vitro					
Reverse mutation (Ames)	*S. typhimurium* TA98, TA100, TA1537	200–5000 µg/plate	Not stated	Negative	Deparade (1988b)
Metabolite CGA 224443					
In vitro					
Reverse mutation (Ames)	*S. typhimurium* TA 98, TA100, TA1535, TA1537 *Escherichia coli* WP2uvrA	20–313 µg/plate	92.6	Negative	Ogorek (1990)
Forward mutation	V79 Chinese hamster lung cells	1.5–40 µg/mL (+S9) 0.2–5.0 µg/mL (–S9)	96.8	Negative	Ogorek (1998)
Chromosomal aberrations	CCL 61 CHO cells	3.1-12.5 µg/mL (+S9) 3.3-13.3 µg/mL (–S9)	92.6	Negative	Strasser (1991)

bw: body weight; CHO: Chinese hamster ovary; DNA: deoxyribonucleic acid; HPRT: hypoxanthine–guanine phosphoribosyltransferase; S9: 9000 × *g* fraction from Aroclor 1254 or phenobarbital/5,6-benzoflavone–induced pretreated rat liver homogenate

the head/trunk in the F_1 males at 250 ppm. Body weights and feed consumption were unaffected by treatment for the P parents. Body weights of the F_1 animals of both sexes at 250 ppm were significantly higher than control values during the premating period. Feed consumption was also slightly higher than control values during this period in the same groups, indicating that the increased body weight was a direct result of increased feed consumption.

For both generations, male and female mating and fertility indices, maternal gestation and parturition indices, and duration of gestation were unaffected by treatment. There were no treatment-related observations at gross necropsy in either generation of adults. No effects on organ weights were seen in the P parents. Organ weights were higher in the 250 ppm male and female F_1 parents, consistent with the higher body weights in this group, but there were no statistically significant differences from control values in the organ-to-body-weight ratios in this group. There were no treatment-related histopathological changes in the reproductive system or in any organs examined in both the P and F_1 parental generations.

In both the F_1 and F_2 offspring, live births, postnatal survival indices and litter weights were unaffected by treatment. The mean time of appearance of the surface righting reflex was slightly delayed by about 0.2–0.4 day in the 250 ppm group compared with controls for both generations. There were no treatment-related observations at postmortem in the F_1 or F_2 offspring.

In this study, the highest dose did not cause any toxicity, a requirement of OECD Test Guideline 416. However, from the 2-year rat study, it is clear that 1500 ppm was above the maximum tolerated dose as animals were euthanized in week 14 because of the extent of tonic–clonic convulsions. In addition, 500 ppm was the lowest-observed-adverse-effect level (LOAEL) based on, for example, tonic–clonic convulsions. A highest dose level of 250 ppm is therefore considered acceptable for this two-generation reproductive toxicity study.

The NOAEL for parental and reproductive effects was 250 ppm (equal to 20.9 mg/kg bw per day for males and 22.2 mg/kg bw per day for females), the highest dose tested.

The NOAEL for offspring toxicity was 100 ppm (equal to 8.3 mg/kg bw per day for males and 8.9 mg/kg bw per day for females), based on the slight delay of 0.2–0.4 day in righting reflex in pups at 250 ppm (equal to 20.9 mg/kg bw per day for males and 22.2 mg/kg bw per day for females) (Fitzgerald & Khalil, 1992).

(b) **Developmental toxicity**

Rats

In a study of developmental toxicity, groups of 25 mated female Sprague Dawley (Crl:CD®(SD) BRVAF/Plus™) rats were administered lufenuron (purity 94.7%) at

a dose of 0, 100, 500 or 1000 mg/kg bw per day in 3% corn starch and 0.5% Tween 80 via oral gavage on gestation day 6 to 15 (inclusive). On gestation day 21, the rats were killed and examined for macroscopic changes in the oral cavity and all organs of the thoracic and abdominal cavities. The ovaries and uterus with cervix were removed, trimmed, weighed intact and examined. Corpora lutea were counted. The uterus was opened and examined for the number and distribution of live fetuses and intrauterine deaths. All fetuses were weighed, sexed and examined for external variations and malformations. Live fetuses were killed, and half of the fetuses from each litter were eviscerated and subsequently processed and examined for skeletal variations and malformations. The remaining fetuses from each litter had the heads removed and fixed in Bouin's fixative for examination of the structure by the serial cross-section technique. The soft tissue of the trunks of these fetuses were internally examined and then eviscerated and processed for skeletal examination.

Twenty-five sperm-positive female rats were assigned to the 0, 100, 500 or 1000 mg/kg bw per day dose levels, of which 22, 23, 19 and 25 animals, respectively, were pregnant. No animals died prior to scheduled kill, and no significant treatment-related signs of toxicity were seen in the individual clinical observations recorded. Minimal maternal toxicity occurred at 1000 mg/kg bw per day, with slight, but statistically significant, reductions in body-weight gain on gestation days 7–9 and in feed consumption on gestation days 6–9.

All pregnant females had live fetuses at termination, and no animals delivered prior to scheduled kill. No embryo/fetal toxicity was apparent at any of the dose levels tested. Statistically significant reductions in the mean number of implantations per dam at 100 mg/kg bw per day and in the number of live fetuses per dam at 100 and 1000 mg/kg bw per day were considered not to be adverse effects of treatment. The reduction at 100 mg/kg bw per day reflected a slight reduction in the number of corpora lutea (developed before treatment), and the less than 4% reduction in mean number of live fetuses at 1000 mg/kg bw per day, compared with concurrent controls, exceeded the mean values for controls from the previous five studies.

The historical control data were presented in the study report as data from control CD rats. No further details were provided; the data were apparently from the performing laboratory, but the exact rat strain is not stated.

There were no significant increases in the test groups, compared with controls, in the occurrences of external, soft tissue or skeletal observations either singularly or collectively when classified as variations or malformations. The mean number of malformed live fetuses in treated groups was similar to control values. Malformations were observed in six, six, three and four fetuses from five, four, two and four litters at 0, 100, 500 and 1000 mg/kg bw per day, respectively. All of these malformations were considered to have occurred spontaneously.

The NOAEL for maternal toxicity was 500 mg/kg bw per day, based on a transient reduction in body-weight gain on gestation days 7–9 and in feed consumption on gestation days 6–9 at 1000 mg/kg bw per day.

The NOAEL for embryo/fetal toxicity was 1000 mg/kg bw per day, the highest dose tested. Lufenuron did not show teratogenic potential in rats (Gilles, 1989).

Rabbits

In a study of developmental toxicity, groups of 16 mated Hra: New Zealand White SPF female rabbits were dosed by gavage at 0, 100, 500 or 1000 mg/kg bw per day with lufenuron (purity 94.7%) in aqueous 3% corn starch and 0.5% Tween 80 on gestation days 7 to 19 (inclusive). The females were monitored daily for clinical signs. Body weights and feed consumption were recorded at selected intervals during gestation. On gestation day 29, the rabbits were killed and examined for macroscopic changes in all organs of the thoracic and abdominal cavities. The ovaries and uterus with cervix were removed, trimmed, weighed intact and examined. Corpora lutea were counted. The uterus was opened and examined for the number and distribution of live fetuses and intrauterine deaths. All fetuses were weighed and examined for external variations and malformations. Each fetus was examined for both soft tissue and skeletal malformations and variations. A transverse section of the brain was made, and organ structure was examined. The soft tissue of the trunks of the fetuses were internally examined. The fetuses were then sexed, eviscerated and processed for skeletal examination.

One female in the control group died on day 18, and one female at 100 mg/kg bw per day was found dead on day 11. Both of these deaths were a result of trauma induced by gavage dosing. Pregnancy was observed at scheduled kill in 13, 15, 12 and 15 females at 0 (controls), 100, 500 and 1000 mg/kg bw per day, respectively. There was no treatment-related effect on fertility. The fertility index was 88%, 100%, 75% and 94% at 0 (controls), 100, 500 and 1000 mg/kg bw per day, respectively. No significant treatment-related signs of toxicity were seen in the individual clinical observations recorded, and there were no treatment-related effects on body weight, body-weight gain or feed consumption. At necropsy, there were no findings that appeared to be treatment related and no effects of treatment on mean absolute or relative organ weights.

All surviving pregnant females had live fetuses on gestation day 29. There were no statistically significant changes in fetal weights or other intrauterine parameters. There was no significant increase, relative to controls, in the occurrence of any external, soft tissue or skeletal malformation or variation in fetuses from does treated with lufenuron.

The NOAEL for maternal toxicity and embryo/fetal toxicity was 1000 mg/kg bw per day, the highest dose tested (Meyer, 1989b).

2.2.6 Special studies

(a) Neurotoxicity

In a repeated-dose neurotoxicity study, groups of 10 male Tif: RAIf (SPF) rats received lufenuron (purity 96.2%) in their diet at 0, 5, 25, 100 or 500 ppm (equal to 0, 0.26, 1.22, 5.43 and 27.0 mg/kg bw per day, respectively) for 4 months. An additional 10 males in the control and high-dose groups served as recovery animals. The investigation was limited to males, as previous studies conducted in rats showed that males were as sensitive as or slightly more sensitive than females to the effects of lufenuron. Animals were observed daily, except for weekends, throughout the study. Detailed clinical observations were not made "blind" (trained observers unaware of the actual treatment group at reading). No data were presented to provide evidence of the ability to detect and quantify, as appropriate, changes in the different end-points recommended for observation, such as autonomic signs, sensory reactivity, limb grip strength and motor activity (use of positive controls). Ophthalmological examinations were not conducted, but data were already available from other studies of similar duration and at similar dose levels. Any observable gross changes were not recorded. The deviations were considered not to compromise the scientific validity of the study. In five animals per dose group, the lufenuron concentrations in fat and blood were determined.

There were no treatment-related deaths during the study. At 500 ppm, single episodes of hyperreactivity to handling, fasciculations and tonic–clonic convulsions were observed in one animal each between weeks 13 and 18. There were no other compound-related clinical signs in the treated animals. Mean body weight and feed consumption were comparable for all groups over the whole study period. No compound-related effects in any of the neurology parameters measured were noted in any group throughout the study period. Motor activity parameters were not affected in any group, and mean startle habituation reactions were comparable for all groups throughout the study. The maze learning error scores did not differ between controls and treated groups at the end of the treatment period or at the end of the recovery period.

Following intraperitoneal injection with 25 mg/kg bw of pentylenetetrazol at the end of the treatment period, generalized tonic–clonic convulsions involving the hindlimbs were induced in top-dose animals only. Comparable motor seizures limited to the head, neck and forelimb areas were induced in all other groups. After a generalized tonic–clonic convulsion, subsequent seizure activity was inhibited, whereas motor seizures were shown frequently throughout the 30-minute observation period. At the end of the recovery period, the incidence

rate for generalized convulsions in top-dose animals was comparable with that seen at the end of the treatment period. Median convulsion scores in the top-dose animals were lower than at the end of the treatment period, but still higher than in control animals, thus indicating partial recovery from the proconvulsive effect induced by the test article.

At the end of the treatment period, the concentrations of lufenuron in fatty tissue were 16, 150, 660 and 2600 mg/kg tissue at 5, 25, 100 and 500 ppm, respectively. Corresponding concentrations in whole blood were 0.1, 0.6, 2.6 and 17 mg/L, indicating a preferential accumulation in fat. In top-dose animals, concentrations of 1600 mg/kg in fat and 4.3 mg/L in blood were measured at the end of the 2-month recovery period.

No treatment-related macroscopic or microscopic changes were seen in the central or peripheral nervous system or in muscles.

The NOAEL for systemic toxicity was 500 ppm (equal to 27.0 mg/kg bw per day), the highest dose tested. The NOAEL for neurotoxicity was 100 ppm (equal to 5.43 mg/kg bw per day), based on spontaneous tonic–clonic convulsions or fasciculations observed in weeks 13–18 and facilitated pentylenetetrazol-induced generalized convulsions at 500 ppm (equal to 27.0 mg/kg bw per day) (Classen, 1992).

(b) **Mechanistic studies**

A study was performed to determine the effects of treatment of lufenuron in the diet for 3 weeks on the estrous cycle in female rats and various plasma hormone levels in male and female rats. Forty-five rats (Crj: Sprague Dawley (SD)) of each sex were randomly allocated to three groups: 0, 500 or 1500 ppm (equal to 0, 30.5 and 92.5 mg/kg bw per day for males and 0, 39.4 and 120.1 mg/kg bw per day for females, respectively). All animals were checked twice a day for mortality and clinical signs. Body weights were recorded at the start of the study and weekly thereafter. Two-day feed consumption per cage was measured weekly, and mean daily feed consumption values per rat were calculated. Vaginal smears were taken each morning from 10 females per group. Proestrous, estrous, metestrous and diestrous stages were determined, and vaginal cytology was examined. Each smear was scored for the relative density of cornified and nucleated cells. At necropsy, 10 mL blood samples were collected under ether anaesthesia from the abdominal aorta of 10 animals per group for analysis of plasma hormone levels (estradiol, progesterone, corticosterone, aldosterone, prolactin, luteinizing hormone, follicle-stimulating hormone [FSH], adrenocorticotropic hormone [ACTH] and testosterone). The left ovaries, adrenals and testes from 10 animals per group were taken to analyse hormone levels in tissue. Gross pathology of these animals consisted of macroscopic examination of the organs and tissues

in the thoracic and abdominal cavities and removal of uterus, ovaries, vagina, testes, adrenals and pituitary for weighing and/or processing for microscopic examination. After 3 weeks of administration of test diet, cholinesterase activity in plasma erythrocyte and brain samples from five rats per group was determined.

No deaths or changes of general condition related to lufenuron administration were observed. With the exception of body-weight gain retardation in the 1500 ppm females at week 3 (96% of controls), there were no differences in mean body weight of either sex between the groups given lufenuron and the control groups. There was no difference between the feed consumption of test and control animals of both sexes.

No statistically significant differences in mean estrous cycle length were found between the treated groups and controls, although there were two cases at 500 ppm of 7 and 10 days' length, respectively, and one case at 1500 ppm of 4.6 days' length, demonstrating prolongation. There was no statistically significant difference in the relative density of cornified and nucleated epithelial cells between the treated and control animals. Statistically significant elevation of mean prolactin, FSH and ACTH levels was noted in males at 1500 ppm. For prolactin, 8/10 animals at 1500 ppm had plasma levels that were within the control range; therefore, the higher mean value for this group was not considered to be biologically significant. The slightly higher FSH levels at 1500 ppm were also not considered large enough to be biologically significant. The increase in ACTH levels is relatively small and may indicate a stress response in these animals that is unrelated to treatment. No other statistically significant differences in hormone levels were found between any of the groups given lufenuron and controls (Table 22).

No gross pathological abnormalities were observed in any of the groups, other than inner dilatation of the uterus in one rat at 1500 ppm. Significantly lower relative uterus weights were noted at 500 ppm compared with controls. This was unlikely to be treatment related, as the effect was slight and there was no dose dependence. There were no statistically significant differences between test and control animals in the weights of the ovaries, testes, adrenals or pituitary.

Angiectasis in the adrenals was observed in one to three female rats in each group and one male rat fed 1500 ppm of lufenuron. Fatty metamorphosis in the adrenals was also noted in one or two male rats in each group. Inner dilatation of the uterus was observed in one rat in each of the control and 1500 ppm groups. No abnormal histopathological findings were noted for the pituitary, ovaries, vagina or testes in any of the groups.

No statistically significant differences in any of the cholinesterase activity samples were noted between the groups of rats given lufenuron and the controls.

The results of this investigation, which focused on the pituitary, adrenal and genital organs, suggest that there is no effect of lufenuron on the endocrine

Table 22
Hormone level data: selected parameters in a 3-week mechanistic study in rats

	Hormone levels					
	Males			Females		
Parameter	**0 ppm**	**500 ppm**	**1 500 ppm**	**0 ppm**	**500 ppm**	**1 500 ppm**
Prolactin (ng/mL)	20.200	22.635	33.420*	85.610	<80.460	95.870
FSH (MIU/mL)	19.4	21.64	22.47**	18.26	16.72	21.50
ACTH (pg/mL)	41.35	50.95	67.88**	115.22	130.57	118.29
Corticosterone (ng/mL)	295.30	325.15	336.46	950.10	977.20	953.30

ACTH: adrenocorticotropic hormone; FSH: follicle-stimulating hormone; IU: International Units; ppm: parts per million; *: $P < 0.05$; **: $P < 0.01$
Source: Tamano (1997)

system in rats of either sex. This conclusion is supported by the reproductive toxicity study in rats, which showed no effect of lufenuron on any reproductive end-point (Tamano, 1997).

2.3 Microbiological effects

Considering the chemical structure and mode of action of lufenuron, the Committee did not anticipate any adverse effects of lufenuron residues on human gastrointestinal microbiota.

2.4 Observations in humans

The sponsor submitted the following information:

Manufacturing employees in Switzerland are medically examined by a company physician at the beginning of their employment and then routinely once a year according to the criteria of the Swiss Accident Insurance Institution (SUVA). Routine medical examinations include:

- anamnesis;
- physical examination including blood pressure;
- blood analysis (haemoglobin, erythrocytes, leukocytes, thrombocytes, complete blood count, blood sedimentation rate, blood sugar, blood pressure, cholesterol, triglycerides, ALAT, aspartate transaminase, ALP, bilirubin, creatinine, uric acid); and
- urine analysis.

The Occupational Health group of Syngenta (formerly ICI/Zeneca Agrochemical) has maintained a database of incidents involving chemical

exposure of workers since 1983. Since 1994, data has been collected on the occurrence of occupationally related illnesses from all Syngenta manufacturing, formulation and packaging sites around the world.

Lufenuron has been handled in large quantities over the last 22 years at a number of sites. No cases of adverse reactions related to the handling of lufenuron have been reported up to July 2015 and no associated adverse health effects have been reported in the workforce.

Lufenuron is used for flea control in companion animals. From 1995 to 1997, 57 incidents of human exposure involving accidental ingestion and/or therapeutic mishaps ("<1/4 tablet" to 409 mg (1.0–26.4 mg/kg)) were reported to two poison centres in the USA. A 15-month-old male vomited within 2 hours of ingestion, without further symptoms, and a 13-year-old female experienced diarrhoea that resolved within 8 hours. The other patients remained asymptomatic for up to 2 hours post ingestion. Lufenuron has shown no significant toxicity in humans from the limited amounts ingested to date (Platform Session 1, 1998).

A breastfeeding mother accidentally ingested an anti-flea preparation of lufenuron. The infant was exposed to an average dose of 0.032 mg/kg bw per day, which is only 3% of the reported acute overdose. No adverse effects were reported during 7 months of follow-up (Bar-Oz, 2000).

3. Comments

3.1 Biochemical aspects

In rats, lufenuron is only partially absorbed following a single oral dose. The extent of absorption is dose related; approximately 20% of a single 100 mg/kg bw dose appears to be absorbed, compared with about 70% of a single 0.1 or 0.5 mg/kg bw dose. A large proportion of the absorbed dose partitions into fat, with very much lower uptake by other tissues, including the brain. All tissue concentrations of radioactivity increased to a maximum 1 day after the last of 14 repeated low doses. The results suggest that most tissue concentrations would plateau within 2–3 weeks of similar repeated dosing. The depot in fat is slowly released, with a terminal half-life of up to 5–13 days at 0.5 mg/kg bw and 10–37 days at 100 mg/kg bw, leading to an increase in concentrations of lufenuron in the brain over long periods (see "Toxicological data", below). Excretion of the absorbed dose is predominantly via faeces; only about 1% of the dose is excreted in urine, independent of the dose. Metabolism of lufenuron is minimal; only about 1% of an oral dose is metabolized by deacylation followed by cleavage of the ureido group. There is no marked sex difference in absorption, tissue distribution, metabolism

or excretion. The pattern of excretion and metabolism is not affected by repeated dosing (Bissig, 1990; Thanei, 1990; Müller, 1995; Okada, 1997; Hassler, 2003; Booth, 2004).

In a study in dogs not previously evaluated by JMPR, no more than 70% of a 10 mg/kg bw oral dose appeared to be absorbed, with C_{max} in blood observed 4 hours after capsule administration. The half-life of lufenuron in blood was estimated to be about 20 days. Judged by the large apparent volume of distribution (19 L/kg), much of the absorbed lufenuron readily distributes into tissues. Up to 54% of the oral dose was excreted via the faeces (mainly during the first 24–72 hours), but only a minor portion (0.8–2.2%) was eliminated in the urine. No parent compound was detected in faeces within 24 hours of administration, but increasing quantities of an unidentified major metabolite and three minor components appeared over the second and third days. Approximately 16% of the administered radioactivity was detected in the skin, 11% in skeletal muscle, 7% in adipose tissue, 1% in liver and bile combined, and 0.3% in blood.

3.2 Toxicological data

Critical toxicity studies are summarized in Table 23.

Lufenuron has low acute toxicity when administered orally or dermally (LD_{50} > 2000 mg/kg bw) or via inhalation (LC_{50} > 2.35 mg/L, maximal attainable concentration) to rats (Hartmann, 1988a,b,c, 1989). Lufenuron was very slightly irritating to the skin and eyes of rabbits and was sensitizing to the skin of guinea-pigs (Schneider, 1988a,b,c).

The most significant toxicological end-point for lufenuron was convulsions, observed after prolonged treatment at high dose levels. Convulsions were observable in all species treated with lufenuron at daily doses of more than 20 mg/kg bw for extended periods (2–3 months in rodents, >3 months in dogs). As lufenuron is a very lipophilic compound (log octanol–water partition coefficient [K_{ow}] = 5.12), it has the potential to accumulate in fatty tissues. Toxicity studies in mice, rats and dogs showed that after prolonged exposure to high doses of 20 mg/kg bw per day or more, fat compartments may become saturated. If exposure is continued after saturation, concentrations in the brain increase, leading to tonic–clonic convulsions.

In a dose range–finding study, lufenuron was fed to mice at a concentration of 0, 1000, 3000 or 9000 mg/kg feed (equal to 0, 151, 449 and 1470 mg/kg bw per day for males and 0, 189, 517 and 1440 mg/kg bw per day for females, respectively) for up to 65 days. As a result of mortality and neurotoxic effects (tonic–clonic seizures) of the test substance at all dose levels, it was concluded

Table 23
Studies relevant to risk assessment

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				
Eighteen-month toxicity and carcinogenicity study (diet)	Males: 0, 0.222, 2.25, 22.6, 62.9 Females: 0, 0.217, 2.12, 22.0, 61.2	Toxicity: mortality, tonic–clonic seizures, increased incidence of fatty liver	2.12	22.0
		Carcinogenicity: None	61.2[a]	–
Rat				
Ninety-day toxicity study (diet)	Males: 0, 1.6, 9.68, 101, 998 Females: 0, 0, 1.7, 10.2, 103, 1 050	Tonic–clonic seizures, increased adrenal weights	9.68	101
Two-year toxicity and carcinogenicity study (diet)	Males: 0, 0.19, 1.93, 20.4, 108 Females: 0, 0.23, 2.34, 24.8, 114	**Tonic–clonic seizures, histopathological effects on lungs, liver, non-glandular stomach, intestines and urinary tract**	1.93 *	20.4
Two-generation reproductive toxicity study (diet)	Males: 0, 0.41, 2.1, 8.3, 20.9 Females: 0, 0.44, 2.2, 8.9 and 22.2	Reproductive toxicity: None	20.9[a]	–
		Parental toxicity: None	20.9[a]	–
		Offspring toxicity: Slight delay in righting reflex	8.3	20.9
Developmental toxicity study (gavage)	0, 100, 500, 1 000	Maternal: Transient reduction in body-weight gain and feed consumption	500	1 000
		Embryo/fetal toxicity: None	1 000[a]	–
Repeated-dose neurotoxicity study (diet)	Males: 0, 0.26, 1.22, 5.43, 27.0	Tonic–clonic seizures	5.43	27.0
Rabbit				
Developmental toxicity study (gavage)	0, 100, 500, 1 000	Maternal toxicity: None	1 000[a]	–
		Embryo/fetal toxicity: None	1 000[a]	–
Dog				
One-year toxicity studies (diet) [b]	Study 1: Males: 0, 3.97, 65.4, 1 880 Females: 0, 3.64, 78.3, 1 980 Study 2: Males: 0, 0.31, 1.42, 7.02, 29.8 Females: 0, 0.33, 1.55, 7.72, 31.8	Toxicity: mortality, convulsions, reduced body-weight gains and histopathological lesions in adrenals, liver, thyroid and lungs and/or clinical chemistry changes	7.02[c]	29.8[d]

* Pivotal study value for the derivation of the ADI (*Bachmann,1993b*)
[a] Highest dose tested.
[b] Two or more studies combined.
[c] Overall NOAEL.
[d] Overall LOAEL.
[e] Lowest dose tested.

that the maximum tolerated dose was exceeded even at 1000 mg/kg feed (equal to 151 mg/kg bw per day) (Fankhauser, 1989a).

In a second dose range–finding study, intended for test substance residue and blood level determination, lufenuron was fed to female mice at a concentration of 0, 4/8, 20, 100 or 1000 mg/kg feed (equal to 0, 0.47/1.1, 2.94, 14.5 and 143 mg/

kg bw per day, respectively; from day 57 onwards, the diet of the low-dose group inadvertently contained 8 mg/kg feed instead of 4 mg/kg feed) for up to 91 days (only 71 days for the high dose). The NOAEL was 100 mg/ kg feed (equal to 14.5 mg/kg bw per day) based on mortality and neurotoxicity (tonic–clonic seizures) at 1000 mg/kg feed (equal to 143 mg/kg bw per day) (Fankhauser, 1990).

In a 28-day range-finding study, rats were administered lufenuron in the diet at a concentration of 0, 50, 400, 3000 or 20 000 mg/kg feed (equal to 0, 4.10, 30.8, 254 and 1690 mg/kg bw per day for males and 0, 4.07, 32.6, 254 and 1740 mg/kg bw per day for females, respectively). The NOAEL was 400 mg/kg feed (equal to 30.8 mg/kg bw per day) based on decreased thymus weight at 3000 mg/ kg feed (equal to 254 mg/kg bw per day) (Fankhauser, 1988).

In a 90-day toxicity study, rats were fed diets containing lufenuron at 0, 25, 150, 1500 or 15 000 mg/kg feed (equal to 0, 1.6, 9.68, 101 and 998 mg/ kg bw per day for males and 0, 1.7, 10.2, 103 and 1050 mg/kg bw per day for females, respectively). The NOAEL was 150 mg/kg feed (equal to 9.68 mg/kg bw per day) based on clinical signs (tonic–clonic seizures), decreased body-weight gain and feed consumption, slight changes in haematology and clinical chemistry parameters and increased adrenal weights at 1500 mg/kg feed (equal to 101 mg/ kg bw per day) (Fankhauser, 1989b).

In a 4-week range-finding study, dogs received lufenuron in their diet at a concentration of 200 or 50 000 mg/kg feed (equal to 8.43 and 2200 mg/kg bw per day for males and 10.1 and 2650 mg/kg bw per day for females, respectively). The NOAEL was 50 000 mg/kg feed (equal to 2200 mg/kg bw per day), the highest dose tested (Briffaux, 1989a).

In a 90-day toxicity study, dogs received lufenuron in their diet at a concentration of 0, 200, 3000 or 50 000 mg/kg feed (equal to 0, 7.8, 122 and 2020 mg/kg bw per day for males and 0, 7.9, 123 and 1930 mg/kg bw per day for females, respectively). The NOAEL was 200 mg/kg feed (equal to 7.8 mg/ kg bw per day) based on increases in blood cholesterol levels and absolute and relative liver weights, reductions in blood potassium and phosphorus levels, and an increase in serum ALP activity in some animals at 3000 mg/ kg feed (equal to 122 mg/kg bw per day) (Briffaux, 1989b).

In a 1-year toxicity study, dogs received lufenuron in their diet at a concentration of 0, 100, 2000 or 50 000 mg/kg feed (equal to 0, 3.97, 65.4 and 1880 mg/kg bw per day for males and 0, 3.64, 78.3 and 1980 mg/kg bw per day for females, respectively). The main target organs were the brain, adrenals, liver, thyroid and lungs. The NOAEL was 100 mg/kg feed (equal to 3.64 mg/ kg bw per day) based on mortality, neuromuscular signs including convulsions, reduced body-weight gain, changes in clinical pathology parameters and histopathological lesions in adrenals, liver, thyroid and lungs observed at 2000 mg/kg feed (equal to 65.4 mg/kg bw per day) (Briffaux, 1992).

In another 1-year toxicity study, dogs received lufenuron in their diet at a concentration of 0, 10, 50, 250 or 1000 mg/kg feed (equal to 0, 0.31, 1.42, 7.02 and 29.8 mg/kg bw per day for males and 0, 0.33, 1.55, 7.72 and 31.8 mg/kg bw per day for females, respectively). The NOAEL was 250 mg/kg feed (equal to 7.02 mg/kg bw per day) based on treatment-related mortality and clinical findings, including convulsions and effects on body weight and on the liver and adrenals, with associated histopathological and/or clinical chemistry changes, at 1000 mg/kg feed (equal to 29.8 mg/kg bw per day) (Altmann, 1995).

An overall NOAEL of 250 mg/kg feed (equal to 7.02 mg/kg bw per day) was identified on the basis of the two 1-year dog studies. The 90-day dog study (Briffaux, 1989b) should not be included in the overall NOAEL as the observed effects (blood parameters and liver weights) were far less severe than the effects in the 1-year dog studies (e.g. mortality) at similar dose levels. This can be explained by the accumulation in fat, which is not yet saturated in the 90-day study; this accumulation leads to higher concentrations of the parent compound in the brain in the longer-term studies.

In an 18-month dietary toxicity and carcinogenicity study, mice received lufenuron at a concentration of 0, 2, 20, 200 or 400 mg/kg feed (equal to 0, 0.222, 2.25, 22.6 and 62.9 mg/kg bw per day for males and 0, 0.217, 2.12, 22.0 and 61.2 mg/kg bw per day for females, respectively). As a result of high mortality in the high-dose group, surviving animals in this dose group were terminated in weeks 9 and 10. The NOAEL was 20 mg/kg feed (equal to 2.12 mg/kg bw per day) based on increased mortality, clinical signs (tonic–clonic convulsive episodes), increased incidences of fatty liver (in females, accompanied by necrotic changes) and a higher incidence of inflammatory changes in the prostate at 200 mg/kg feed (equal to 22.0 mg/kg bw per day). No treatment-related tumours were observed (Bachmann, 1993a).

In a 2-year dietary toxicity and carcinogenicity study, rats received lufenuron at a concentration of 0, 5, 50, 500 or 1500 mg/kg feed (equal to 0, 0.19, 1.93, 20.4 and 108 mg/kg bw per day for males and 0, 0.23, 2.34, 24.8 and 114 mg/ kg bw per day for females, respectively). As a result of overt toxicity at 1500 mg/ kg feed, all animals in this group were terminated in week 14. The NOAEL was 50 mg/kg feed (equal to 1.93 mg/kg bw per day) based on clinical signs (tonic–clonic convulsions), decreased body weight, and (histo)pathological effects on lungs, liver, non-glandular stomach, intestines and urinary tract at 500 mg/ kg feed (equal to 20.4 mg/kg bw per day). No treatment-related tumours were observed (Bachmann, 1993b).

The Committee concluded that lufenuron is not carcinogenic in mice or rats.

Lufenuron was tested for genotoxicity in an adequate range of assays, both in vitro and in vivo. No evidence of genotoxicity was found.

The Committee concluded that lufenuron is unlikely to be genotoxic.

In view of the lack of genotoxicity and the absence of carcinogenicity in mice and rats, the Committee concluded that lufenuron is unlikely to pose a carcinogenic risk to humans.

In a two-generation reproductive toxicity study, rats received lufenuron in their diet at a concentration of 0, 5, 25, 100 or 250 mg/kg feed (equal to 0, 0.41, 2.1, 8.3 and 20.9 mg/kg bw per day for males and 0, 0.44, 2.2, 8.9 and 22.2 mg/kg bw per day for females, respectively, based on mean intakes for combined P and F1 generations during the premating period). The NOAEL for parental and reproductive effects was 250 mg/kg feed (equal to 20.9 mg/kg bw per day), the highest dose tested. The NOAEL for offspring toxicity was 100 mg/kg feed (equal to 8.3 mg/kg bw per day) based on the slight delay in righting reflex in pups at 250 mg/kg feed (equal to 20.9 mg/kg bw per day) (Fitzgerald & Khalil, 1992).

In a study of developmental toxicity, rats were administered lufenuron via gavage at a dose of 0, 100, 500 or 1000 mg/kg bw per day. The NOAEL for maternal toxicity was 500 mg/kg bw per day based on a transient reduction in body-weight gain on gestation days 7–9 and feed consumption on gestation days 6–9 at 1000 mg/kg bw per day. The NOAEL for embryo/fetal toxicity was 1000 mg/kg bw per day, the highest dose tested. (Gilles, 1989).

In a study of developmental toxicity, rabbits were dosed at 0, 100, 500 or 1000 mg/kg bw per day with lufenuron via gavage. The NOAEL for maternal toxicity and embryo/fetal toxicity was 1000 mg/kg bw per day, the highest dose tested (Meyer, 1989b).

The Committee concluded that lufenuron is not teratogenic.

In a repeated-dose neurotoxicity study, male rats received lufenuron in their diet at 0, 5, 25, 100 or 500 mg/kg feed (equal to 0, 0.26, 1.22, 5.43 and 27.0 mg/kg bw per day, respectively) for 4 months. No systemic toxicity was observed. The NOAEL for neurotoxicity was 100 mg/kg feed (equal to 5.43 mg/kg bw per day) based on spontaneous tonic–clonic convulsions or fasciculations observed in weeks 13–18 and facilitated pentylenetetrazol-induced generalized convulsions at 500 mg/kg feed (equal to 27.0 mg/kg bw per day) (Classen, 1992).

Convulsions were observed in all species after prolonged treatment with lufenuron, owing to saturation of the accumulation in fatty tissues, with subsequent increased lufenuron levels in the brain. The neurotoxic effects are not expected to occur after a single dose.

The Committee concluded that lufenuron is not acutely neurotoxic, but is neurotoxic after prolonged treatment.

A study was performed to determine the effects of 3-week lufenuron treatment on the estrous cycle in female rats and various plasma hormone levels (estradiol, progesterone, corticosterone, aldosterone, prolactin, luteinizing hormone, FSH, ACTH and testosterone) in male and female rats administered

a dietary concentration of 0, 500 or 1500 mg/kg feed (equal to 0, 30.5 and 92.5 mg/kg bw per day for males and 0, 39.4 and 120.1 mg/kg bw per day for females, respectively). The results of this investigation, focused on the pituitary, adrenal and genital organs, suggest that there is no effect of lufenuron on the endocrine system in rats of either sex (Tamano, 1997). This conclusion is supported by the reproductive toxicity study in rats, which showed no effect of lufenuron on any reproductive end-point (Fitzgerald & Khalil, 1992).

No specific studies on immunotoxicity were submitted. The available repeated-dose studies do not indicate an immunotoxic potential for lufenuron following exposure by the oral route.

3.3 Microbiological data

Considering the chemical structure and mode of action of lufenuron, the Committee did not anticipate any adverse effects of lufenuron residues on human gastrointestinal microbiota.

3.4 Observations in humans

In reports on manufacturing plant personnel, no adverse health effects were noted. Several incident reports indicate no significant toxicity in humans.

The Committee concluded that the existing database on lufenuron was adequate to characterize the potential hazards to the general population, including fetuses, infants and children.

4. Evaluation

An ADI of 0–0.02 mg/kg bw was established on the basis of the NOAEL of 1.93 mg/kg bw per day for tonic–clonic seizures and findings in lungs, gastrointestinal tract, liver and urinary tract in the 2-year dietary study in rats, using a safety factor of 100.

The Committee concluded that it was not necessary to establish an acute reference dose for lufenuron 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 for lufenuron there are no specific concerns for less-than-lifetime exposure.

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Appendix 1

Historical control data for 2-year rat study

Table A-1

Historical control data for benign interstitial cell tumour of testis in the 2-year rat study

Laboratory database				RITA database for SD rats, 1985–1995, 2-year oral studies			
Study no.	Date of first dose	Incidence of benign interstitial cell tumours	No. of testes examined	Study no.	Date of first dose	Incidence of benign interstitial cell tumours	No. of testes examined
785271	Oct 78	2	90	1	Mar 85	4	68
801440	Mar 81	6	80	40	Sep 88	2	50
800218	Jun 82	1	58	45	Jul 89	2	55
820872	Aug 82	1	79	46	Jul 86	2	49
820650	Apr 83	2	80	47	Feb 89	4	71
82648	Oct 83	0	79	50	Sep 89	1	55
821482	Oct 83	2	79	52	Jul 86	1	50
820559	Feb 84	1	80	58	Nov 87	0	50
821638	Apr 84	3	70	62	Aug 87	6	60
830732	Nov 84	2	79	70	Jul 86	2	48
840171	Mar 85	4	78	96	Nov 93	4	60
850335	Feb 86	6	80	101	Oct 92	0	55
850853	May 86	1	80	102	Oct 92	1	55
874076	May 87	1	50	106	Nov 93	1	60
860087	Sep 87	3	80	111	Jul 95	5	59
850703	Jan 88	1	79	140	Oct 94	1	60
876047	May 88	2	70	141	Oct 94	2	60
861139	Feb 89	4	80	–	–	–	–
861154	Mar 89	0	80	–	–	–	–
886178	Aug 89	2	70	–	–	–	–
901463	Dec 90	4	80	–	–	–	–
891326	Feb 91	1	60	–	–	–	–
901483	May 92	2	60	–	–	–	–
911123	Aug 92	0	60	–	–	–	–

no.: number; RITA: Registry of Industrial Toxicology Animal-data; SD: Sprague Dawley

Table A-2

Historical control data (laboratory database) for benign granular cell tumour of the cerebral meninges in the 2-year rat study

Study no.	Date of first dose	Incidence of benign granular cell tumours in the meninges	No. of organs examined
785271	Oct 78	0	88
800218	Jun 82	0	60
801440	Mar 81	0	79
820559	Feb 84	0	80
820648	Oct 83	1	78
820650	Apr 83	0	80
820872	Aug 82	0	80
821482	Oct 83	1	79
821638	Apr 84	1	68
830732	Nov 84	1	77
840171	Mar 85	1	78
850703	Jan 88	3	80
860087	Sep 87	3	80
861139	Feb 89	4	80
861154	Mar 89	1	80
874076	May 87	2	50
876047	May 88	2	70
886178	Aug 89	0	70

no.: number

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).
5. Evaluation of the carcinogenic hazards of food additives (Fifth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 29, 1961; WHO Technical Report Series, No. 220, 1961 (out of print).
6. Evaluation of the toxicity of a number of antimicrobials and antioxidants (Sixth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 31, 1962; WHO Technical Report Series, No. 228, 1962 (out of print).
7. Specifications for the identity and purity of food additives and their toxicological evaluation: emulsifiers, stabilizers, bleaching and maturing agents (Seventh report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 35, 1964; WHO Technical Report Series, No. 281, 1964 (out of print).
8. Specifications for the identity and purity of food additives and their toxicological evaluation: food colours and some antimicrobials and antioxidants (Eighth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 38, 1965; WHO Technical Report Series, No. 309, 1965 (out of print).
9. Specifications for identity and purity and toxicological evaluation of some antimicrobials and antioxidants. FAO Nutrition Meetings Report Series, No. 38A, 1965; WHO/Food Add/24.65 (out of print).
10. Specifications for identity and purity and toxicological evaluation of food colours. FAO Nutrition Meetings Report Series, No. 38B, 1966; WHO/Food Add/66.25.

11. Specifications for the identity and purity of food additives and their toxicological evaluation: some antimicrobials, antioxidants, emulsifiers, stabilizers, flour treatment agents, acids, and bases (Ninth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 40, 1966; WHO Technical Report Series, No. 339, 1966 (out of print).

12. Toxicological evaluation of some antimicrobials, antioxidants, emulsifiers, stabilizers, flour treatment agents, acids, and bases. FAO Nutrition Meetings Report Series, No. 40A, B, C; WHO/Food Add/67.29.

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.

14. Specifications for the identity and purity of food additives and their toxicological evaluation: some flavouring substances and non nutritive sweetening agents (Eleventh report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 44, 1968; WHO Technical Report Series, No. 383, 1968.

15. Toxicological evaluation of some flavouring substances and non nutritive sweetening agents. FAO Nutrition Meetings Report Series, No. 44A, 1968; WHO/Food Add/68.33.

16. Specifications and criteria for identity and purity of some flavouring substances and non-nutritive sweetening agents. FAO Nutrition Meetings Report Series, No. 44B, 1969; WHO/Food Add/69.31.

17. Specifications for the identity and purity of food additives and their toxicological evaluation: some antibiotics (Twelfth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 45, 1969; WHO Technical Report Series, No. 430, 1969.

18. Specifications for the identity and purity of some antibiotics. FAO Nutrition Meetings Series, No. 45A, 1969; WHO/Food Add/69.34.

19. Specifications for the identity and purity of food additives and their toxicological evaluation: some food colours, emulsifiers, stabilizers, anticaking agents, and certain other substances (Thirteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 46, 1970; WHO Technical Report Series, No. 445, 1970.

20. Toxicological evaluation of some food colours, emulsifiers, stabilizers, anticaking agents, and certain other substances. FAO Nutrition Meetings Report Series, No. 46A, 1970; WHO/Food Add/70.36.

21. Specifications for the identity and purity of some food colours, emulsifiers, stabilizers, anticaking agents, and certain other food additives. FAO Nutrition Meetings Report Series, No. 46B, 1970; WHO/Food Add/70.37.

22. Evaluation of food additives: specifications for the identity and purity of food additives and their toxicological evaluation: some extraction solvents and certain other substances; and a review of the technological efficacy of some antimicrobial agents (Fourteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 48, 1971; WHO Technical Report Series, No. 462, 1971.

23. Toxicological evaluation of some extraction solvents and certain other substances. FAO Nutrition Meetings Report Series, No. 48A, 1971; WHO/Food Add/70.39.

24. Specifications for the identity and purity of some extraction solvents and certain other substances. FAO Nutrition Meetings Report Series, No. 48B, 1971; WHO/Food Add/70.40.

25. A review of the technological efficacy of some antimicrobial agents. FAO Nutrition Meetings Report Series, No. 48C, 1971; WHO/Food Add/70.41.

26. Evaluation of food additives: some enzymes, modified starches, and certain other substances: Toxicological evaluations and specifications and a review of the technological efficacy of some antioxidants (Fifteenth report

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27. Toxicological evaluation of some enzymes, modified starches, and certain other substances. FAO Nutrition Meetings Report Series, No. 50A, 1972; WHO Food Additives Series, No. 1, 1972.

28. Specifications for the identity and purity of some enzymes and certain other substances. FAO Nutrition Meetings Report Series, No. 50B, 1972; WHO Food Additives Series, No. 2, 1972.

29. A review of the technological efficacy of some antioxidants and synergists. FAO Nutrition Meetings Report Series, No. 50C, 1972; WHO Food Additives Series, No. 3, 1972.

30. Evaluation of certain food additives and the contaminants mercury, lead, and cadmium (Sixteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 51, 1972; WHO Technical Report Series, No. 505, 1972, and corrigendum.

31. Evaluation of mercury, lead, cadmium and the food additives amaranth, diethylpyrocarbamate, and octyl gallate. FAO Nutrition Meetings Report Series, No. 51A, 1972; WHO Food Additives Series, No. 4, 1972.

32. Toxicological evaluation of certain food additives with a review of general principles and of specifications (Seventeenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 53, 1974; WHO Technical Report Series, No. 539, 1974, and corrigendum (out of print).

33. Toxicological evaluation of some food additives including anticaking agents, antimicrobials, antioxidants, emulsifiers, and thickening agents. FAO Nutrition Meetings Report Series, No. 53A, 1974; WHO Food Additives Series, No. 5, 1974.

34. Specifications for identity and purity of thickening agents, anticaking agents, antimicrobials, antioxidants and emulsifiers. FAO Food and Nutrition Paper, No. 4, 1978.

35. Evaluation of certain food additives (Eighteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 54, 1974; WHO Technical Report Series, No. 557, 1974, and corrigendum.

36. Toxicological evaluation of some food colours, enzymes, flavour enhancers, thickening agents, and certain other food additives. FAO Nutrition Meetings Report Series, No. 54A, 1975; WHO Food Additives Series, No. 6, 1975.

37. Specifications for the identity and purity of some food colours, enhancers, thickening agents, and certain food additives. FAO Nutrition Meetings Report Series, No. 54B, 1975; WHO Food Additives Series, No. 7, 1975.

38. Evaluation of certain food additives: some food colours, thickening agents, smoke condensates, and certain other substances. (Nineteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 55, 1975; WHO Technical Report Series, No. 576, 1975.

39. Toxicological evaluation of some food colours, thickening agents, and certain other substances. FAO Nutrition Meetings Report Series, No. 55A, 1975; WHO Food Additives Series, No. 8, 1975.

40. Specifications for the identity and purity of certain food additives. FAO Nutrition Meetings Report Series, No. 55B, 1976; WHO Food Additives Series, No. 9, 1976.

41. Evaluation of certain food additives (Twentieth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Food and Nutrition Meetings Series, No. 1, 1976; WHO Technical Report Series, No. 599, 1976.

42. Toxicological evaluation of certain food additives. WHO Food Additives Series, No. 10, 1976.

43. Specifications for the identity and purity of some food additives. FAO Food and Nutrition Series, No. 1B, 1977; WHO Food Additives Series, No. 11, 1977.

44. Evaluation of certain food additives (Twenty-first report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 617, 1978.

45. Summary of toxicological data of certain food additives. WHO Food Additives Series, No. 12, 1977.

46. Specifications for identity and purity of some food additives, including antioxidant, food colours, thickeners, and others. FAO Nutrition Meetings Report Series, No. 57, 1977.

47. Evaluation of certain food additives and contaminants (Twenty-second report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 631, 1978.

48. Summary of toxicological data of certain food additives and contaminants. WHO Food Additives Series, No. 13, 1978.

49. Specifications for the identity and purity of certain food additives. FAO Food and Nutrition Paper, No. 7, 1978.

50. Evaluation of certain food additives (Twenty-third report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 648, 1980, and corrigenda.

51. Toxicological evaluation of certain food additives. WHO Food Additives Series, No. 14, 1980.

52. Specifications for identity and purity of food colours, flavouring agents, and other food additives. FAO Food and Nutrition Paper, No. 12, 1979.

53. Evaluation of certain food additives (Twenty-fourth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 653, 1980.

54. Toxicological evaluation of certain food additives. WHO Food Additives Series, No. 15, 1980.

55. Specifications for identity and purity of food additives (sweetening agents, emulsifying agents, and other food additives). FAO Food and Nutrition Paper, No. 17, 1980.

56. Evaluation of certain food additives (Twenty-fifth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 669, 1981.

57. Toxicological evaluation of certain food additives. WHO Food Additives Series, No. 16, 1981.

58. Specifications for identity and purity of food additives (carrier solvents, emulsifiers and stabilizers, enzyme preparations, flavouring agents, food colours, sweetening agents, and other food additives). FAO Food and Nutrition Paper, No. 19, 1981.

59. Evaluation of certain food additives and contaminants (Twenty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 683, 1982.

60. Toxicological evaluation of certain food additives. WHO Food Additives Series, No. 17, 1982.

61. Specifications for the identity and purity of certain food additives. FAO Food and Nutrition Paper, No. 25, 1982.

62. Evaluation of certain food additives and contaminants (Twenty-seventh report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 696, 1983, and corrigenda.

63. Toxicological evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 18, 1983.

64. Specifications for the identity and purity of certain food additives. FAO Food and Nutrition Paper, No. 28, 1983.

65. Guide to specifications – General notices, general methods, identification tests, test solutions, and other reference materials. FAO Food and Nutrition Paper, No. 5, Rev. 1, 1983.

66. Evaluation of certain food additives and contaminants (Twenty-eighth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 710, 1984, and corrigendum.

67. Toxicological evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 19, 1984.

68. Specifications for the identity and purity of food colours. FAO Food and Nutrition Paper, No. 31/1, 1984.

69. Specifications for the identity and purity of food additives. FAO Food and Nutrition Paper, No. 31/2, 1984.

70. Evaluation of certain food additives and contaminants (Twenty-ninth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 733, 1986, and corrigendum.

71. Specifications for the identity and purity of certain food additives. FAO Food and Nutrition Paper, No. 34, 1986.

72. Toxicological evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 20. Cambridge University Press, 1987.

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77. Evaluation of certain food additives and contaminants (Thirty-first report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 759, 1987, and corrigendum.

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80. Evaluation of certain veterinary drug residues in food (Thirty-second report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 763, 1988.

81. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series, No. 23. Cambridge University Press, 1988.

82. Residues of some veterinary drugs in animals and foods. FAO Food and Nutrition Paper, No. 41, 1988.

83. Evaluation of certain food additives and contaminants (Thirty-third report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 776, 1989.

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87. Residues of some veterinary drugs in animals and foods. FAO Food and Nutrition Paper, No. 41/2, 1990.

88. Evaluation of certain food additives and contaminants (Thirty-fifth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 789, 1990, and corrigenda.

89. Toxicological evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 26, 1990.

90. Specifications for identity and purity of certain food additives. FAO Food and Nutrition Paper, No. 49, 1990.

91. Evaluation of certain veterinary drug residues in food (Thirty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 799, 1990.

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ANNEX 2

Abbreviations used in the monographs

ACT	Australian Capital Territory
ACTH	adrenocorticotropic hormone
ADI	acceptable daily intake
ADME	absorption, distribution, metabolism, and excretion
AFC	alveolar foam cells
ALAT or ALT	alanine aminotransferase [also alanine transaminase]
ALP	alkaline phosphatase
APTT	activated partial thromboplastin time
ARfD	acute reference dose
AST	aspartate transaminase [also aspartate aminotransferase]
ATSDR	Agency for Toxic Substances and Disease Registry
AUC	area under the concentration–time curve
AUC_{0-tn}	area under concentration–time curve up to last nonzero value
AUC_{oral}	area under concentration–time curve after oral administration
$AUC_{i.v.}$	area under concentration–time curve after intravenous administration
BUN	blood urea nitrogen
bw	body weight
CAS	Chemical Abstracts Service
CCRVDF	Codex Committee on Pesticide Residues of Veterinary Drugs in Foods
CHO	Chinese hamster ovary
CHQ	5-chloroquinolin-8-ol
CIA	Critically Important Antimicrobials [for Human Medicine]
CK	creatinine kinase
5-CL	5-chloroquinolin-8-ol
CLSI	Clinical and Laboratory Standards Institute
C_{max}	maximum concentration / peak concentration
CNS	central nervous system
CYP	cytochrome P450
DCHQ	5,7 dichloroquinolin-8-ol
df	degrees of freedom
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
ECG	electrocardiogram

eq.	equivalents
EUCAST	European Committee on Antimicrobial Susceptibility Testing
F	female
FAO	Food and Agriculture Organization of the United Nations
FOB	functional observational battery
FSH	follicle-stimulating hormone
GGT	γ-glutamyltranspeptidase (or γ-glutamyltransferase)
GLP	good laboratory practice
GSD	geometric standard deviation
HLT	hypocellularity of lymphatic tissue
HPLC	high-performance liquid chromatography
HPP	hypocellularity of Peyer's patch
HPRT	hypoxanthine–guanine phosphoribosyltransferase
ICHQ	5-chloro-7-iodo-quinolin-8-ol
IU	International Units
IUPAC	International Union of Pure and Applied Chemistry
IV or i.v.	intravenous
JECFA	Joint FAO/WHO Expert Committee on Food Additives
JMPR	Joint FAO/WHO Meeting on Pesticide Residues
KCP	Kupffer cell pigmentation
LC_{50}	median lethal concentration
LC-MS	liquid chromatography–mass spectrometry
LC-MS/MS	liquid chromatography coupled with tandem mass spectrometry
LD_{50}	median lethal dose
LDH	lactate dehydrogenase
LDPE	low-density polyethylene
LLOQ	lower limit of quantification
LOAEL	lowest-observed-adverse-effect level
LOQ	limit of quantification / level of quantitation
mADI	microbiological acceptable daily intake
max.	maximum
MCHC	mean cell haemoglobin concentration
MCV	mean cell volume / mean corpuscular volume
MIC	minimum inhibitory concentration
MIC_{50}	minimum concentration required to inhibit the growth of 50% of organisms
MIC_{90}	minimum concentration required to inhibit the growth of 90% of organisms
min.	minimum
MMAD	mass median aerodynamic diameter
MNPCE / MPE	micronucleated polychromatic erythrocytes

MNU	*N*-methyl-*N*-nitrosourea
MRL	maximum residue limit
MRT	mean residence time
MTD	maximum tolerated dose
n.a. / NA	not applicable
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NE	normochromatic erythrocyte
no. / No.	number
NOAEC	no-observed-adverse-effect concentration
NOAEL	no-observed-adverse-effect level
OECD	Organisation for Economic Co-operation and Development
OECD TG	Organisation for Economic Co-operation and Development Test Guideline
PCE or PE	polychromatic erythrocyte
PEG	polyethylene glycol
PEG400	polyethylene glycol 400
ppm	parts per million
RBC	red blood cell
RD_{50}	50% respiratory rate decrease
RITA	Registry of Industrial Toxicology Animal-data
S9	9000 × *g* supernatant fraction from liver homogenate from induced animals [metabolic activation]
SD / std dev	standard deviation
$t_{½}$	half-life
T_{max}	time to reach the maximum concentration (C_{max})
TK	thymidine kinase
TLC	thin-layer chromatography
TOCP	tri-*o*-cresyl phosphate
USEPA	United States Environmental Protection Agency
USFDA	United States Food and Drug Administration
v/v	volume per volume
VICH	International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products
w/v	weight per volume
WBC	white blood cell
WHO	World Health Organization

ANNEX 3

Joint FAO/WHO Expert Committee on Food Additives[1]

Geneva, 17–26 October 2017

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ANNEX 4

Recommendations on the substances on the agenda

Amoxicillin (antimicrobial agent)

Acceptable daily intake	The Committee established an mADI of 0–0.002 mg/kg body weight (bw) based on the effects of amoxicillin on the intestinal microbiota.
Acute reference dose	The Committee established an ARfD of 0.005 mg/kg bw based on microbiological effects on the intestinal microbiota.
Estimated chronic dietary exposure	The GECDE for the general population is 0.14 µg/kg bw per day, which represents 7% of the upper bound of the mADI.
Estimated acute dietary exposure	The GEADE for the general population is 1.4 µg/kg bw, which represents 28% of the microbiological ARfD. The GEADE for children is 1.6 µg/kg bw, which represents 31% of the microbiological ARfD.
Residue definition	Amoxicillin

Recommended maximum residue limits (MRLs)

Species	Fillet[a] (µg/kg)	Muscle (µg/kg)
Finfish[b]	50	50

[a] Muscle plus skin in natural proportion.
[b] The term "finfish" includes all fish species.

Ampicillin (antimicrobial agent)

Acceptable daily intake	The Committee established an overall mADI of 0–0.003 mg/kg bw based on a NOAEL equivalent to 0.025 mg/kg bw per day for increase in population(s) of ampicillin-resistant bacteria in the gastrointestinal tract in humans, and using a safety factor of 10 (for the variability in the composition of the intestinal microbiota within and between individuals).
Acute reference dose	The Committee established an ARfD of 0.012 mg/kg bw based on the microbiological end-point.

Estimated chronic dietary exposure	The GECDE for the general population is 0.29 µg/kg bw per day, which represents 10% of the upper bound of the ADI.
Estimated acute dietary exposure	The GEADE for the general population is 1.9 µg/kg bw per day, which represents 16% of the ARfD. The GEADE for children is 1.7 µg/kg bw per day, which represents 14% of the ARfD.
Residue definition	Ampicillin
Maximum residue limits	The Committee recommended an MRL of 50 µg/kg for ampicillin in finfish muscle and in finfish muscle plus skin in natural proportion, the same as that recommended for amoxicillin, because the modes of action, the physicochemical properties and the toxicological and pharmacokinetic profiles of amoxicillin and ampicillin are very similar

Ethion (acaricide)

Acceptable daily intake	The Committee established an ADI of 0–0.002 mg/kg bw based on the NOAEL of 0.2 mg/kg bw per day for embryotoxic effects in a rat developmental toxicity study, and using a safety factor of 100 (10 for interspecies variability and 10 for intraspecies variability).
Acute reference dose	The Committee established an ARfD of 0.02 mg/kg bw based on the NOAEL of 0.15 mg/kg bw for erythrocyte acetylcholinesterase inhibition in a repeated-dose human study, and using an intraspecies safety factor of 10.
Estimated dietary exposure	No dietary exposure assessment could be conducted.
Residue definition	None. A suitable marker residue could not be determined and a marker to total residue ratio could not be established.
Maximum residue limits	The Committee was unable to recommend MRLs for ethion.

Flumethrin (type II pyrethroid insecticide)

Acceptable daily intake	The Committee established an ADI of 0–0.004 mg/kg bw based on the NOAEL of 0.37 mg/kg bw per day for skin lesions in parental animals and reduced survival and body-weight gain in pups in a twogeneration toxicity study in rats, and using a safety factor of 100 (10 for interspecies variability and 10 for intraspecies variability).
Acute reference dose	The Committee established an ARfD of 0.005 mg/kg bw based on the NOAEL of 0.5 mg/kg bw for salivation in dams in a developmental toxicity study in rats, and using a safety factor of 100 (10 for interspecies variability and 10 for intraspecies variability).
Estimated chronic dietary exposure	As flumethrin is also used as pesticide, the overall dietary exposure was estimated. The assumptions and detailed results will be presented in the JECFA 85 report. The results below are only for the use of flumethrin as a veterinary drug. The GECDE for the general population is 0.008 µg/kg bw per day, which represents 0.2% of the upper bound of the ADI. The GECDE for children is 0.006 µg/kg bw per day, which represents 0.2% of the upper bound of the ADI.
Estimated acute dietary exposure	The GEADE for the general population is 0.1 µg/kg bw per day, which represents 2.2% of the ARfD. The GEADE for children is 0.1 µg/kg bw per day, which represents 2.2% of the ARfD.
Residue definition	Flumethrin (*trans*-Z1 and *trans*-Z2 diastereomers at a ratio of approximately 60:40).
Maximum residue limits	The Committee set an MRL for honey of 6 µg/kg, which is twice the limit of quantification (LOQ; 3 µg/kg) of the most reliable analytical method (liquid chromatography coupled with tandem mass spectrometry; LC-MS/MS) used in the residues studies.

Lufenuron (insecticide)

Acceptable daily intake	The Committee established an ADI of 0–0.02 mg/kg bw based on the NOAEL of 1.93 mg/kg bw per day for tonic–clonic seizures and findings in lungs, gastrointestinal tract, liver and urinary tract in a 2-year dietary study in rats, and using a safety factor of 100 (10 for interspecies variability and 10 for intraspecies variability).
Acute reference dose	The Committee concluded that it was unnecessary to establish an ARfD for lufenuron in view of its low acute oral toxicity and the absence of developmental toxicity and other toxicological effects likely to be elicited by a single dose.
Estimated chronic dietary exposure	As lufenuron is also used as pesticide, the overall dietary exposure was estimated. The assumptions and detailed results will be presented in the JECFA 85 report. The results below are only for the use of lufenuron as a veterinary drug. The GECDE for the general population is 1.1 µg/kg bw per day, which represents 5.5% of the upper bound of the ADI.
Residue definition	Lufenuron

Recommended maximum residue limits (MRLs)[a]

Species	Fillet (µg/kg)
Salmon	1 350
Trout	1 350

[a] There were insufficient zilpaterol residue data to adequately consider exposure to residues in lungs and other edible offal of cattle apart from liver and kidney.

Monepantel (anthelminthic)

Acceptable daily intake	The ADI of 0–0.02 mg/kg bw per day established by the Committee at the seventy-fifth meeting (WHO TRS No. 969, 2012) remains unchanged.
Acute reference dose	The Committee concluded that it was unnecessary to establish an ARfD.
Estimated dietary exposure	The GECDE for the general population is 13.7 µg/kg bw per day, which represents 68% of the upper bound of the ADI.

	The GECDE for children is 5.0 µg/kg bw per day, which represents 25% of the upper bound of the ADI. The GECDE for infants is 4.4 µg/kg bw per day, which represents 22% of the upper bound of the ADI.
Residue definition	Monepantel sulfone

Recommended maximum residue limits (MRLs)[a]

Species	Fat (µg/kg)	Kidney (µg/kg)	Liver (µg/kg)	Muscle (µg/kg)
Cattle	7 000	1 000	2 000	300

[a] Determined as monepantel sulfone, expressed as monepantel.

Sisapronil (ectoparasiticide)

No additional data were submitted. As a result, the ADI remains unestablished.

Zilpaterol hydrochloride (β2-adrenoceptor agonist)

Following evaluation of the bioavailability data submitted, the MRLs recommended by the Committee at its eighty-first meeting (WHO TRS No. 997, 2016) remain unchanged.

This volume contains monographs prepared at the eighty-fifth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), which met in Geneva, Switzerland, from 17 to 26 October 2017.

The toxicological monographs in this volume summarize data on the veterinary drug residues that were evaluated toxicologically by the Committee: amoxicillin, ampicillin, ethion, flumethrin, halquinol and lufenuron. Annexed to the report is a summary of the Committee's recommendations on these and other drugs discussed at the eighty-fifth 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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