



RESIDUE EVALUATION OF CERTAIN VETERINARY DRUGS

Joint FAO/WHO Expert Committee on Food Additives

85th meeting 2017



Food and Agriculture
Organization of the
United Nations



World Health
Organization

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FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS

Rome, 2018

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Most of the evaluations and summaries contained in this publication are based on unpublished proprietary data submitted to JECFA for use when making its assessment. A registration authority should not consider granting a registration based on an evaluation published herein unless it has first received authorization for such use from the owner of the data or any second party that has received permission from the owner for using the data.

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Geneva, 17–26 October 2017

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Abbreviations

ADI	Acceptable daily intake
ADME	Absorption, distribution, metabolism and excretion
ARfD	acute reference dose
AUC	area under the curve
bw	bodyweight
CAC	Codex Alimentarius Commission
CAS	Chemical Abstracts Service
CC β	detection capability
CCRVDF	Codex Committee on Residues of Veterinary Drugs in Foods
Cl _T	total body clearance
C _{max}	maximum concentration (plasma)
CV	coefficient of variation
CVMP	Committee for Medicinal Products for Veterinary Use, European Medicines Agency
d	day
DAD	diode array detector (in HPLC)
EC	electrochemical detection (liquid chromatography)
EDI	estimated daily intake
EMA	European Medicines Agency (formerly EMEA)
EMEA	European Agency for the Evaluation of Medicinal Products
eq	equivalent
ESI	electrospray interface (in LC/MS and LC-MS/MS)
FAO	Food and Agriculture Organization of the United Nations
GC	gas chromatography
GC-MS	gas chromatography/mass spectrometry
GCP	good clinical practice
GEADE	global estimate of acute dietary exposure
GECDE	global estimate of chronic dietary exposure
GLP	good laboratory practice
GVP	good veterinary practice
h	hour

HPLC/FL	high performance liquid chromatography with fluorescence detection
i.m.	intramuscular [injection]
INN	International Non-proprietary Name
IT	ion trap (mass spectrometer)
IUPAC	International Union of Pure and Applied Chemistry
i.v.	intravenous [injection]
JECFA	Joint FAO/WHO Expert Committee on Food Additives
JMPR	Joint FAO/WHO Meeting on Pesticide Residues
kg	kilogram (10^3 g)
L	litre
LC	liquid chromatography
LC-MS	high performance liquid chromatography/mass spectrometry
LC-MS/MS	high performance liquid chromatography/tandem mass spectrometry
LLOQ	lower limit of quantification
LOAEL	lowest observed adverse effect level
LOD	limit of detection
LOQ	limit of quantification
LSC	liquid scintillation counter
µg	microgram (10^{-6} g)
mg	milligram (10^{-3} g)
min	minimum or minute
mL	millilitre
mM	milliMolar
MR	marker residue
MRL	maximum residue limit
MRT	mean residence time
MS	mass spectrometry
MSPD	matrix solid phase dispersion
MW	molecular weight
m/z	mass to charge ratio
ND	not detectable

NOAEL	no observed adverse effect level
NQ	non-quantifiable
NS	Not sampled/ no sample analysed
PLE	pressurized liquid extraction
QC	quality control
QuEChERS	Quick, Easy, Cheap, Effective, Rugged and Safe
rsd	relative standard deviation
rpm	revolutions per minute
SD	standard deviation
SPE	solid phase extraction
$t_{1/2}$	half life
$T_{1/2\beta}$	plasma elimination half-life
$T_{1/2K_a}$	half-life of distribution rate constants
t_{max}	time to peak plasma concentration
TLC	thin layer chromatography
TMDI	theoretical maximum daily intake
TR	total residue
TRR	total radiolabelled residues
ULOQ	upper limit of quantification
USDA	United States Department of Agriculture
UV	ultraviolet
$V_{d(ss)}$	volume of distribution at steady state
VICH	International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products
WHO	World Health Organization

Introduction

This volume of FAO JECFA Monographs contains residue evaluation of certain veterinary drugs prepared at the 85th Meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), held in Geneva, Switzerland, 17–26 October 2017. This was the twenty-fifth meeting of JECFA convened specifically to consider residues of veterinary drugs in food-producing animal species. The tasks for the Committee were to further elaborate principles for evaluating the safety of residues of veterinary drugs in food and for establishing acceptable daily intakes (ADIs) and/or acute reference doses (ARfDs), and to recommend maximum residue limits (MRLs) for substances on the agenda when they are administered to food-producing animals in accordance with good veterinary practice in the use of veterinary drugs. The enclosed monographs provided the scientific basis for the recommendations of MRLs.

Background

In response to the growing use of veterinary medicines in food animal production systems internationally and the potential implications for human health and fair trading practices, a Joint FAO/WHO Expert Consultation on Residues of Veterinary Drugs was convened in Rome in November 1984 (FAO/WHO, 1985). One of the major recommendations of this consultation was the establishment of the Codex Committee on Residues of Veterinary Drugs in Foods (CCRVDF) and the periodic convening of an appropriate expert body to provide independent scientific advice to this Committee and to member countries of FAO and WHO. At its first session, in Washington, DC, in November 1986, the CCRVDF reaffirmed the need for such a scientific body and made a number of recommendations and suggestions to be considered by JECFA (CCRVDF, 1986). In response to these recommendations, the 32nd JECFA meeting was devoted entirely to the evaluation of residues of veterinary drugs in food - a new responsibility for the Joint FAO/WHO Expert Committee on Food Additives.

85th Meeting of JECFA

The present volume contains monographs on the evaluations of residue data of seven substances scheduled for evaluation at the 85th Meeting of the Committee: amoxicillin, ampicillin, ethion, halquinol, lufenuron and monepantel.

The monographs are prepared in a uniform format consistent with the data provided and the specific request for risk assessment by CCRVDF. The format includes identity of substance, residues in food and their evaluation, metabolism studies, tissue residue depletion studies, methods of residue analysis, a final appraisal of the study results, and if appropriate, recommendations on MRLs. A summary of the recommendations on compounds on the agenda and further information required is included in Annex 1. In addition, a summary of JECFA evaluations of residues of veterinary drugs in foods from the 32nd meeting to the present 85th meeting can be found in Annex 2.

The monographs of this volume must be considered in the context of the full report of the meeting, which will be published in the *WHO Technical Report Series*.

On-line editions of *Residues of some veterinary drugs in animals and foods* are available online at <http://www.fao.org/food/food-safety-quality/scientific-advice/jecfa/jecfa-vetdrugs/en/>. The search interface is available in five languages (Arabic, Chinese, English, French and Spanish) and allows searching for compounds, functional classes, ADI and MRL status.

Contact and feedback

More information on the work of the Committee is available from FAO at <http://www.fao.org/food/food-safety-quality/scientific-advice/jecfa/en/>.

Please send questions and feedback to jecfa@fao.org.

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Addendum to the monograph prepared by the 75th meeting of the Committee and published in the FAO Food & Nutrition Papers/FAO JECFA Monographs.

Background

Amoxicillin (CAS N°. 26787-78-0) is a broad-spectrum, pharmacologically active beta-lactam antibiotic effective against Gram-positive and Gram-negative bacteria. It is available as sodium salt that is very soluble in water, and a trihydrate salt that is less soluble in water (Park *et.al.*, 2016). Amoxicillin is a widely used antibiotic in human and veterinary medicine for the treatment and control of respiratory, gastrointestinal, urinary and skin bacterial infection due to its pharmacological and pharmacokinetic properties.

Amoxicillin was previously evaluated by the Committee as its Seventy-fifth Meeting (FAO, 2011) which established an acceptable daily intake (ADI) of 0–0.7 µg/kg body weight (bw) based on the basis of microbiological effects. The Committee recommended MRLs for amoxicillin in cattle, sheep and pig tissue of 50 µg/kg and in cattle and sheep milk of 4 µg/kg, determined as amoxicillin parent compound.

The Committee did not calculate an EDI for amoxicillin 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 liter of milk with the MRLs recommended above, the theoretical maximum daily intake (TMDI) is 31 µg/person per day, which represents 74 % of the upper bound of the ADI.

The 23rd session of the CCRVDF requested that JECFA recommend MRLs in fin fish muscle and skin in natural proportions.

Current evaluation

A Member State submitted a set of data including information on three amoxicillin formulations, list of trade names and approved uses in fish, information of labels from amoxicillin commercial products, monitoring data of amoxicillin residues in aquaculture products (Anonymous, 2015), and the English translation of a report containing studies on pharmacology and residue depletion for amoxicillin in fish (Park *et.al.*, 2013). Additionally, four publications, translated from their original language, were submitted for review: Seo *et.al.*,

2014, Jeon *et.al.*, 2010, Chung *et.al.*, 2006 and Son *et.al.*, 2011. The sponsor provided some of the raw data requested.

A comprehensive literature search of information related to amoxicillin available in public databases was conducted to complement the information submitted by the sponsor. The search was conducted using the following databases: Agricola, Embase, Web of Science, PubMed, Springer Protocols, Food Science and Technology Abstracts, Phish-Pharm, CABI VetMed Resource, and included the years from when the databases were created until when the search was concluded.

The following criteria were applied to filter the articles regarding the assessment to be conducted:

Inclusion criteria	Exclusion criteria
Any article focusing on: <ul style="list-style-type: none"> - Amoxicillin concentrations in plasma of fish - Amoxicillin concentrations in edible tissues of fish - Residue determination in fish plasma / tissue - Bioavailability of Amoxicillin residues in fish No restrictions concerning year of publication	Any article focusing on: <ul style="list-style-type: none"> - Bacteria resistance to Amoxicillin - Amoxicillin use in food animal species other than fish - Kinetics/residues of antimicrobials other than Amoxicillin (and do not include Amoxicillin for comparison) Any article dealing with ornamental fish Any article focusing on environmental issues

The terms used during the searches and dates when the searches were finalized are listed below:

- Amoxicillin AND fish AND residue (search completed by April 18, 2017)
- Amoxicillin AND fish AND kinetics (search completed by April 18, 2017)
- Amoxicillin AND fish AND withdrawal (search completed by April 18, 2017)
- Amoxicillin AND fish AND metabolism (search completed by May 24, 2017)
- Amoxicillin AND fish AND analytical method (search completed by June 5, 2017)
- Amoxicillin AND (salmon OR trout) AND (residue OR kinetics OR withdrawal OR metabolism OR analytical method) (search completed June 5, 2017)

The literature search resulted in 37 potentially relevant articles, and a detailed review of the articles listed above determined the exclusion of 12 articles. Twenty-five articles remained as relevant and their data were used in the current monograph.

More papers were identified to be potentially relevant, but had been excluded as they were only available in their original language and no English translation was available. Tu *et.al.*, 2006 (cobia), Tsukahara *et.al.*, 1989 (yellowtail), Jo *et.al.*, 2006 (fish meat), Kim *et.al.*, 2010

(residues of antibiotic in fish), Kim *et.al.*, 2015 (olive flounder), Nakauchi *et.al.*, 1989 (yellowtail) were some of the publications that seemed to be relevant for the evaluation, however as they were only available in their original language, they were not considered in this document. The only exception was the Tsukahara *et.al.*, (1989) paper that refers to residues of amoxicillin in yellowtail, and from which information from the abstract and some tables were used in this monograph for comparison of values.

Residues in food and their evaluation

Conditions of use

Literature available in the public domain describes that amoxicillin has been reported to provide good results against furunculosis in Atlantic salmon (Inglis *et.al.*, 1992, 1993; Brown and Grant, 1992) and pseudotuberculosis in yellowtail (Nakauchi *et.al.*, 1989).

In a Member State, veterinary medicines containing amoxicillin are registered for the treatment and prevention of fish disease. Amoxicillin hydrate is used for the treatment of furunculosis (*Photobacterium damsela* subsp. *piscicida*) in yellowtail. Amoxicillin sodium is used in olive flounder for prevention of Streptococcosis (*Streptococcus iniae*). An association of amoxicillin (tri)hydrate in combination with florfenicol is used for prevention of Streptococcosis (*Streptococcus iniae*) and Edwardsiellosis (*Edwardsiella tarda*) in olive flounder. Additionally, literature available in the public domain describes that in a Member State, amoxicillin is licensed for use in fisheries, orally administered mixed with feed for the treatment of pasteurellosis or abscesses (Manual for Animal-use Pharmaceuticals; MAP, 2001; Jeon *et.al.*, 2010). Also, in Member State fisheries sector is reporting the use of amoxicillin for gram positive and gram negative bacteria, pseudotuberculosis and furunculosis, in cultured fish (Son *et.al.*, 2011).

Since 2013, single intramuscular dose of 40 mg of amoxicillin sodium/kg bw fish has been approved for treating streptococcosis of olive flounder in a Member State (Lim *et.al.*, 2016 sourcing from National Fisheries Research and Development Institute, NFRDI, <http://www.nifs.go.kr>).

In a Member State, the maximum residue limit (MRL) of amoxicillin used in fisheries is set at 50 µg/ kg for all fish and shellfish.

In another Member State, amoxicillin has been approved for treatment of fish disease for salmon, eel, perciform and other fish, with an MRL of 50 µg/kg for edible tissues.

In a different Member State, MRLs were set for all food producing species, including fin fish: MRL of 50 µg/kg for edible tissues and 4 µg/kg for milk.

Dosage

In one Member State, amoxicillin hydrate is administered by oral route at dose of 20 - 40 mg/kg bw fish, once or twice a day, for a period of 4 to 7 days in yellowtail against furunculosis. The optimum dose is 40 mg/kg bw and the higher dose is 80 mg/kg bw of fish. Based on the dose regimen approved in this Member State for yellowtail, amoxicillin is administered to flounder, rockfish and red sea bream.

Amoxicillin sodium is administered by intramuscular route as a dose of 12.5 or 40 mg/kg bw of fish, once a day, in olive flounder, against streptococcosis (*Streptococcus iniae*).

An association of Amoxicillin (tri)hydrate in combination with florfenicol is administered as upper intramuscular injection at a single dose of 10 mg/kg in olive flounder (*Paralichthys olivaceus*), against Streptococcus and Edwardsiella.

The submission did not provide any recommended withdrawal period.

In another Member State, amoxicillin has been approved for administration by oral route at a dose of 40 mg/kg.

Pharmacokinetics and metabolism

Pharmacokinetics in Food-producing Animals

The pharmacokinetics of amoxicillin in pre-ruminating and ruminating cattle, lactating dairy cows, pigs and sheep, including lactating sheep have been evaluated at the 75th meeting of the Committee. Metabolism studies were limited and two metabolites, amoxicilloic acid and amoxicillin piperazine 2'-5'-dione, were described in pigs.

No absorption, distribution, metabolism and excretion (ADME) studies in fish or other species using radiolabelled amoxicillin were provided. No metabolism studies of amoxicillin in fish were submitted.

Fish

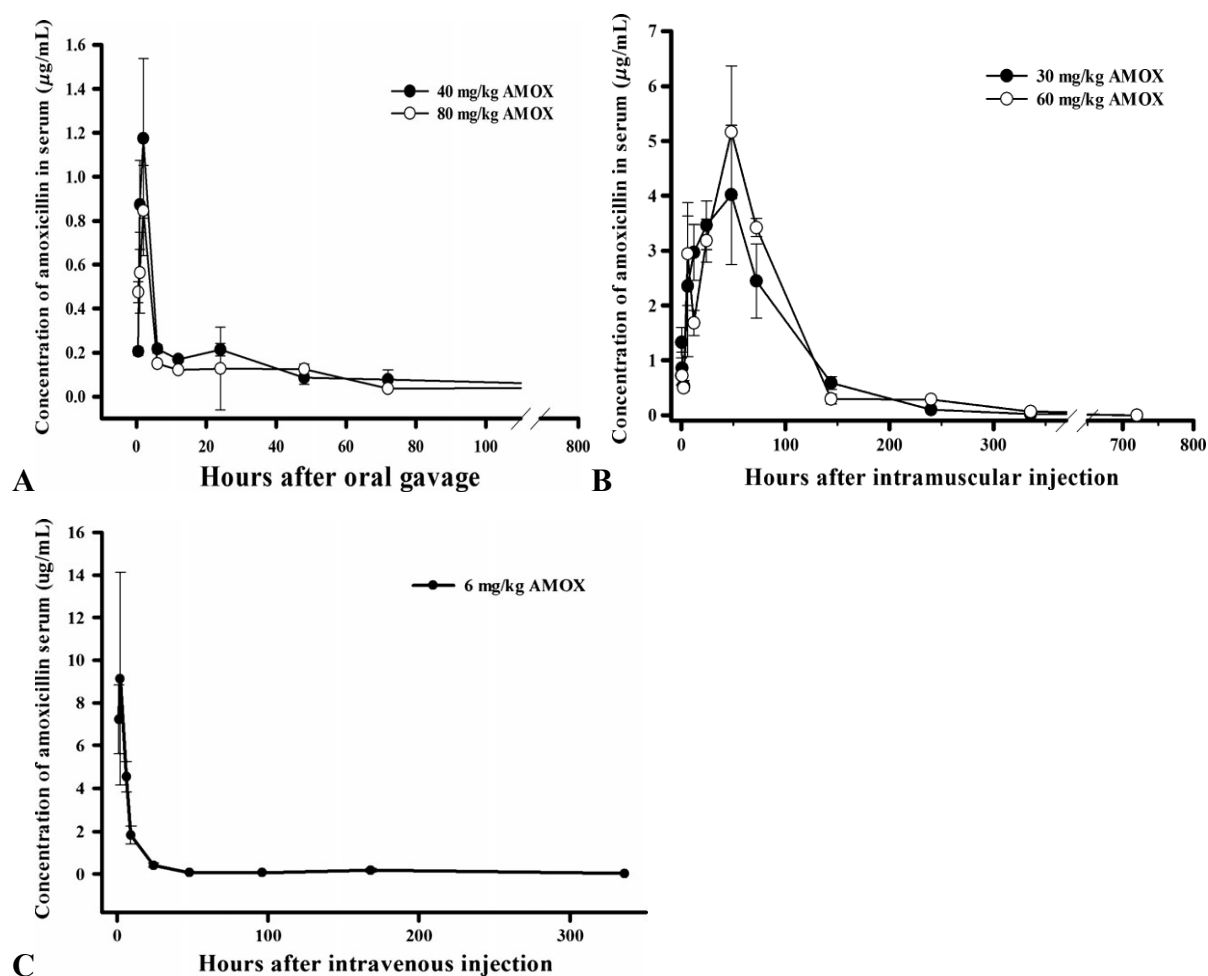
Eleven studies investigating the pharmacokinetics of amoxicillin in fish were submitted for evaluation or have been found in the public domain. The studies include six different types of fish: eel (Jeon *et.al.*, 2010), flatfish (Park, *et.al.*, 2013), sea bream (Della Rocca *et.al.*, 2004), pompano (Wang *et.al.*, 2015a), salmon (Brown and Grant, 1992; Inglis *et.al.*, 1992; Inglis *et.al.*, 1993), and olive flounder (Seo *et.al.*, 2014; Park *et.al.*, 2016; Lim *et.al.*, 2016). Three of these studies were conducted with amoxicillin sodium (Park *et.al.*, 2016; Lim *et.al.*, 2016) and the rest with amoxicillin trihydrate. None of these studies are reported as GLP studies. Studies are briefly described below and are listed per the fish species tested.

Olive flounder

A study was conducted to investigate the pharmacokinetics of amoxicillin trihydrate in olive flounder (*Paralichthys olivaceus*) after oral, intramuscular and intravenous administration (Seo *et.al.*, 2014). Four hundred fish (average weight of 500 ± 20 g) randomly divided into five tanks were acclimatized at a constant temperature (20 ± 1.5 °C) for 2 weeks before amoxicillin

administration. They were maintained at that temperature, with a flow-through of filtered seawater for the 30 days of study duration. Using a modified syringe, the fish previous lightly anesthetized, received the amoxicillin containing feed directly into the stomach to a final single dose of 40 and 80 mg/kg bw per day. In another group of animals, doses of 30 and 60 mg/kg bw per day of amoxicillin were administered by a single i.m injection in the back muscle. In a different group a dose of 6 mg/kg bw of amoxicillin per day was administered into the fish caudal vein under deep anesthesia with MS-222. Five fish were collected, at each time interval, on the first day at 0, 0.5, 1, 2, 6, 12, and 24 h following administration and 2, 3, 6, 10, 14, 20, and 30 days thereafter. Plasma samples were separated by centrifugation and stored at -80 °C until analysis. Serum samples were analyzed using high-performance liquid chromatography. Calibration curve was linear with high correlation coefficients ($r^2 > 0.995$). The recoveries from plasma were of 75 %, 80 %, and 85 % for 0.5, 1, and 10 µg/ml, respectively. The LOD was estimated to be 0.03 µg/ml and LOQ, 0.1 µg/ml. During stability studies, samples stored at -80 °C showed no significant difference until 2 months. After oral, i.m. and i.v. administration, amoxicillin plasma concentration-time curves were obtained and are showed in Figure 1 A, B and C, respectively.

Figure 1. Amoxicillin concentrations in serum after oral (A), intramuscular (B) and intravenous (C) administration. (Seo *et.al.*, 2014)



Oral amoxicillin administration of 40 mg/kg, resulted in a C_{\max} of $1.1 \pm 0.1 \mu\text{g/mL}$, at 1.7 h, with a rapidly decreased concentration of $0.22 \pm 0.01 \mu\text{g/mL}$ at 6 h, reaching $0.03 \pm 0.002 \mu\text{g/mL}$ after 30 days from administration. With a dose of 80 mg/kg of amoxicillin, the C_{\max} resulted of $0.7 \pm 0.3 \mu\text{g/mL}$ at 1.6 h with a rapidly decreased concentration to $0.15 \pm 0.01 \mu\text{g/mL}$ at 6 h. The C_{\max} values observed after oral dose treatment are not proportional to the dose administrated. Also, although early concentration–time dose of amoxicillin was altered by the increase of oral dose administrated, there was no difference in amoxicillin plasma concentrations measured after 48 h of administration. The C_{\max} values observed after i.m. administration of 30 and 60 mg/kg of amoxicillin, were $4.0 \pm 0.51 \mu\text{g/mL}$ at 29 h and $4.3 \pm 0.1 \mu\text{g/mL}$ at 38 h after dose, respectively. Following i.v injection of 6 mg/kg bw of amoxicillin, the C_{\max} values resulted of $9.1 \pm 0.34 \mu\text{g/mL}$ at 2.1 h but declining to $0.4 \mu\text{g/mL}$ at 24 h and $0.05 \mu\text{g/mL}$ at 20 days of administration. The bioavailability for amoxicillin was calculated to be of 9 % and 3.6 % in olive flounder, after oral administration of 40 and 80 mg/kg, respectively, and of 86 and 53 % following i.m administration of 30 and 60 mg/kg of amoxicillin, respectively. The kinetic parameters are described in Table 1.

Table 1. Pharmacokinetic parameters of amoxicillin trihydrate in olive flounder calculated using two-compartment model, after oral, intramuscular, and intravenous administration (Seo *et.al.*, 2014)

Parameter	Unit	Oral administration		Intramuscular administration		Intravenous injection
		40 mg/kg	80 mg/kg	30 mg/kg	60 mg/kg	6 mg/kg
AUC	$\mu\text{g.h/mL}$	52.23	41.22	370.27	543.65	86.27
$T_{1/2\beta}$	H	259.1	285.6	39.3	27.5	97.0
t_{\max}	H	1.70	1.64	29.8	39.6	2.17
C_{\max}	$\mu\text{g/mL}$	1.1	0.7	4.0	4.3	9.1

AUC, area under the concentration–time curve; $T_{1/2\beta}$, half-life associated with the macro constant beta (elimination phase); t_{\max} , time to peak plasma concentration; C_{\max} , peak plasma concentration.

A different study was conducted to determine the pharmacokinetics of an injectable amoxicillin sodium formulation in olive flounder (*Paralichthys olivaceus*) (Park *et.al.*, 2016). Two hundred male and female fish (average weight of $140 \pm 22 \text{ g}$ and age range 150–180 d) were acclimatized for 2 weeks in fresh water at $23 \pm 1 \text{ }^{\circ}\text{C}$. Two treatment groups were randomly selected to receive an intramuscular dose of either 12.5 mg/kg or 125 mg/kg amoxicillin sodium. Blood samples were taken from the caudal vein at 0, 1, 2, 4, 8, 12, 24, and 48 h after administration, store at $-20 \text{ }^{\circ}\text{C}$ until they were analyzed by high-performance liquid chromatography (HPLC). Analytical performances of the HPLC method (linearity, accuracy, recovery, precision, LOD and LOQ) were determined and the method was validated. HPLC method for analysis of amoxicillin in serum samples was specific and sensitive. Calibration curve was linear with high correlation ($r^2 > 0.997$). A recovery for serum was found to range from 97 to 99 %; LOD was calculated as $0.01 \mu\text{g/mL}$ and LOQ $0.04 \mu\text{g/mL}$. PK parameters for male and female olive flounder were combined because no gender-dependent differences were observed. Figure 2 shows the amoxicillin concentrations in serum after i.m. administration of 12.5 mg/kg bw and

125 mg/kg bw amoxicillin sodium. Bars represent standard deviations. Table 2 lists the PK parameters of amoxicillin in olive flounder that were calculated by non-compartmental methods. Amoxicillin showed a rapid absorption from i.m. site, with average peak concentrations (C_{\max}) of 22.05 mg/ml and 106.76 mg/ml reached at 2.6 h and 2.2 h after administration of 12.5 mg/kg and 125 mg/kg doses, respectively. A linear dose–exposure relationship was evidenced, in which, for a dose of 125 mg/kg dose, a 10-fold ($2755.37 \text{ h} \times \text{mg/ml}$) increase was detected compared with the average $\text{AUC}_{0-24 \text{ h}}$ after 12.5 mg/kg dose ($273.69 \text{ h} \times \text{mg/ml}$). For the 12.5 mg/kg and 125 mg/kg dose, the average terminal half-lives were 15.52 and 10.42 h, respectively. PK profile shows rapid absorption, prolonged action duration (as evidenced by long terminal half-life and MRT values) and dose-proportional increases in exposure (AUC)

Figure 2. Semi-logarithmic plot of serum concentration-time course of amoxicillin after treatment with a single i.m. dose of amoxicillin sodium at 12.5 mg/kg or 125 mg/kg bw to olive flounder (n=40 per group). Bars represent standard deviations. (Park. *et.al.*, 2016)

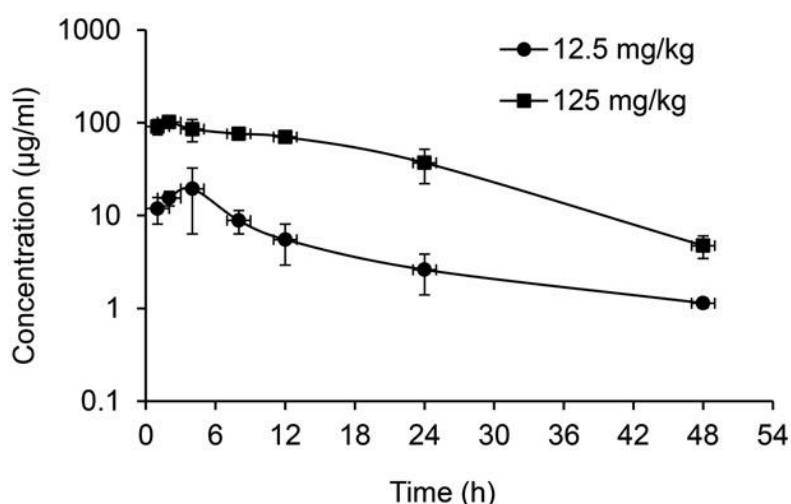


Table 2. Amoxicillin PK parameters after an intramuscular dose of amoxicillin sodium at 12.5 mg/kg or 125 mg/kg to olive flounder. (Park. *et.al.*, 2016)

Parameter	Unit	Intramuscular administration	
		12.5 mg/kg	125 mg/kg
t_{\max}	h	2.60 ± 1.34	2.20 ± 1.09
C_{\max}	mg/ml	22.05 ± 11.09	106.76 ± 18.03
$t_{1/2\text{L}}$	h	15.52 ± 3.86	10.42 ± 0.95
AUC	mg.h/ml	273.69 ± 48.80	2755.37 ± 588.15
MRT	h	18.79 ± 4.49	14.44 ± 2.67

t_{\max} , time of peak drug concentration; C_{\max} , peak drug concentration; AUC, area-under-the-serum concentration versus time curve from zero to time infinity; $t_{1/2\text{L}}$, terminal half-life; MRT, mean residence time.

Another study was conducted to determine amoxicillin concentrations in plasma samples collected from olive flounder (*Paralichthys olivaceus*) treated with a single intramuscular (i.m.) dose of either 40 or 80 mg/kg of amoxicillin sodium (Lim *et.al.* 2016). Seven healthy olive

flounder (average weight of 821 ± 125 g) per tank were acclimatized for one week in filtered seawater maintained at $22 \text{ }^{\circ}\text{C} \pm 1 \text{ }^{\circ}\text{C}$. Blood samples were collected from the caudal vein of seven fish per time point at 0.5, 1, 3, 6, 12, 24, 36, 48, 72, and 96 h after amoxicillin dose, processed and stored at $-80 \text{ }^{\circ}\text{C}$ until analysis by HPLC. Amoxicillin calibration curve for HPLC-UV was linear in the range of 0.01-100 $\mu\text{g/ml}$ ($r^2 = 0.9999$); the LOD and LOQ was calculated to be 0.18 and 0.54 $\mu\text{g/ml}$, respectively. Amoxicillin average recoveries from plasma after doses of 1 and 10 $\mu\text{g/ml}$ were 96.9 and 95.8 %, respectively. Amoxicillin was rapidly absorbed following a single intramuscular dose of either 40 mg/kg bw or 80 mg/kg bw. After the lower dose, the peak plasma concentration (C_{max}) was of 62.64 $\mu\text{g/ml}$ and the time to peak plasma concentration (t_{max}) was 1.59 h. After the single i.m dose of 80 mg/kg bw, the C_{max} was 87.61 $\mu\text{g/ml}$ and t_{max} 3.02 h. Pharmacokinetics parameters are listed in Table 3. Amoxicillin mean plasma concentrations as function of time after a single i.m amoxicillin dose, were best described by a one-compartment pharmacokinetic model (Figure 3).

Figure 3. Amoxicillin plasma concentrations (log) vs time, after olive flounder treatment with a single i.m. dose of either 40 or 80 mg/kg bw (Lim *et.al.*, 2016)

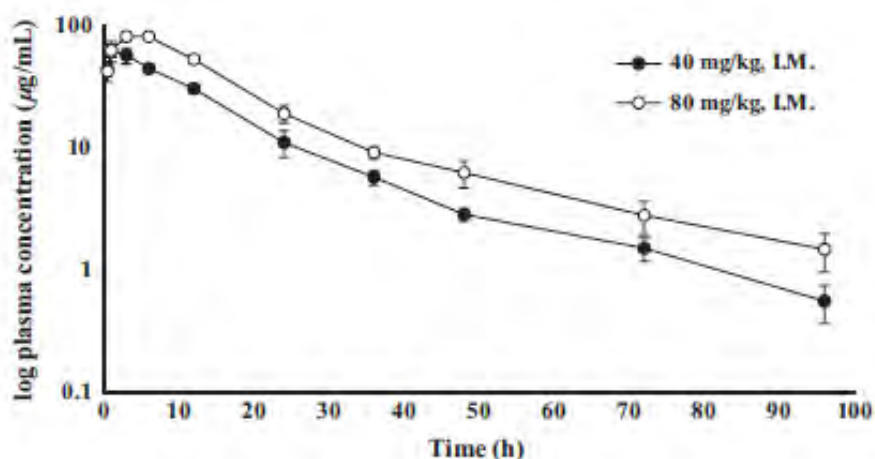


Table 3. Pharmacokinetic parameter of amoxicillin sodium obtained from olive flounder treated with a single i.m. amoxicillin dose of either 40 or 80 mg/kg bw. (Lim *et.al.*, 2016)

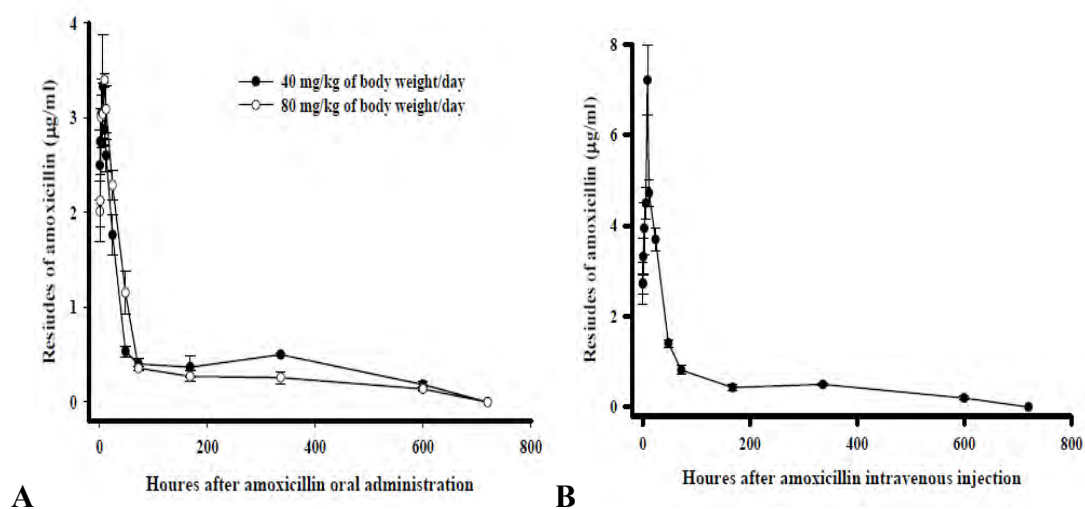
Parameter	Unit	40 mg/kg	80 mg/kg
t_{max}	H	1.59	3.02
C_{max}	$\mu\text{g/mL}$	62.64	87.61
AUC	$\mu\text{g/mL} \times \text{h}$	933.23	1592.55
MRT	H	13.67	15.89

t_{max} , time to peak plasma concentration; C_{max} , peak plasma concentration; AUC, area under the concentration–time curve from zero to time infinity; MRT, mean residence time.

Eel

A study was conducted to investigate the pharmacokinetic properties of amoxicillin trihydrate in eel, *Anguilla japonica* (average weight of 220 ± 10 g), after either single oral administration (40 or 80 mg/kg bw) or single intravenous injection (i.v.) of 1 mg/kg bw (Jeon *et.al.*, 2010). The animals were divided in groups of 80 individuals per each experimental group, acclimatized at a constant temperature (28 ± 1.5 °C) for 2 weeks before amoxicillin administration, and maintained at that temperature in a flow-through water tank through the duration of the study. Oral dosing was delivered in feed (animals were not fed the day before) and the i.v. dose was administrated in the caudal vein of the fish while there were lightly anesthetized. Blood samples from each group were collected at times 0 (immediately following administration), 0.5h, 1h, 3h, 6h, 9h, 12h, 24h (1 day), 48h (2 days), 96h (3 days), 168h (7 days), 336h (14 days), 720h (30 days), from 5 animals each time interval. The separated plasma samples were stored at -80 °C until the time of analysis. Samples were analyzed using high-performance liquid chromatography method with UV detection, with a limit of detection (LOD) estimated to be 0.1 µg/ml and limit of quantitation (LOQ), 0.2 µg/ml. The amoxicillin recovery from plasma at 0.5 , 1 and 10 µg/ml was on average of 75, 80 and 85 %, respectively. Amoxicillin plasma concentration-time curves were obtained after oral and i.v. administration and they are shown in Figure 4 A and B, respectively.

Figure 4. Amoxicillin concentrations in serum after oral (A) and intravenous (B) administration. (Jeon *et.al.*, 2010)



The study group receiving the 40 mg/kg oral dose resulted in the highest blood concentration of 3.3 ± 0.5 µg/ml at 2.1 h after amoxicillin administration, remaining at $0.53 \pm 0.1 - 0.1 \pm 0.1$ µg/ml from 48 h to 20 days, and then decreasing below 0.05 µg/ml after 25 days. After 3.6 h of amoxicillin administration, the group receiving the 80 mg/kg oral dose showed the highest blood concentration of 3.4 ± 0.1 µg/ml, but rapidly decreasing to $0.3 \pm 0.1 - 0.1 \pm 0.1$ µg/ml after 72 h. The maximum amoxicillin plasma concentrations did not increase proportionally (same C_{max} value with the two oral doses tested) to the amoxicillin concentration administrated orally. The concentration increased linearly to reach the maximum concentration

during the initial h after administration, but after 48 h, no particular correlation to the concentration of administration was found. After 9 h of amoxicillin administration by a single intravenous injection at a concentration of 1 mg/kg a maximum plasma concentration of 4.2 ± 0.8 µg/ml was reached, declining to 1.4 ± 0.1 at 48 h, with a trend of drug elimination to 0.1 ± 0.1 µg/ml after 20 days. The bioavailability for amoxicillin was calculated to be of 1.6 % and 1.1 % in *A. japonica*, following oral administration of 40 and 80 mg/kg, respectively. Bioavailability for amoxicillin in eel, *A. japonica*, was low. The kinetic parameters are described in Table 4.

Table 4. Pharmacokinetic parameters of amoxicillin trihydrate in *A. japonica*, calculated using two-compartment model, after oral (A) and intravenous (B) administration. (Jeon *et.al.*, 2010)

A. Oral administration

Parameter	Unit	40 mg/kg	80 mg/kg
AUC	µg × h/mL	464	667
T _{1/2} K _a	h	0.4	0.6
T _{1/2} β	h	868	3139
t _{max}	h	2.1	3.6
C _{max}	µg/mL	3.4	3.4

AUC, Area under a concentration of analyte vs. time curve from zero to time infinity; T_{1/2}K_a half-life of distribution rate constants; T_{1/2}β, terminal half-life; t_{max}, The time of peak concentration; C_{max}, The peak or maximum concentration.

B. Intravenous administration

Parameter	Unit	1 mg/kg
AUC	µg.h/mL	748
T _{1/2} β	h	2989
MRT	h	2527

AUC, Area under a concentration of analyte vs. time curve from zero to time infinity; T_{1/2}β, Terminal half-life associated; MRT, mean residence time

Flatfish

A study was conducted to determine the amoxicillin concentration in plasma after administering Kamoxin (amoxicillin sodium) in flatfish muscle and reported in a technical report (Park, *et.al.*, 2013). Fish, weighting 150 g, were maintained in water temperature $22 \pm 3^\circ\text{C}$, continuous aeration, pH 6.5-8.5, and DO around 5.0-6.0 ppm. They were injected with 5, 10, 20, and 40 mg/kg of amoxicillin in the dorsal muscle. At 0, 15 min., 30 min., 1, 3, 6, 12, 24, 48, 72, and 96 h after treatment, 5 fish were slaughtered, plasma separated and analyzed by HPLC. The pharmacokinetic variables were analyzed with a non-compartmental model. After 30 minutes to 1 hour of amoxicillin administration, the maximum concentration of amoxicillin residue in plasma, is reached. After reaching the equilibrium state, elimination half-life is about 15-20 h. Following amoxicillin administration dose of 10 mg/kg, blood concentration reached maximum in 15 minutes (9.53 ± 2.42 µg/ml), decreasing to 3.51 ± 1.25 µg/ml after 6 h (Figure 5A). After amoxicillin dose administration of 20 mg/kg, the maximum concentration

in plasma was reached in one hour ($35.34 \pm 2.21 \mu\text{g/ml}$), decreasing to $13.42 \pm 2.76 \mu\text{g/ml}$ after 6 h (Figure 5B). With a 40 mg/kg amoxicillin dose, a concentration of at least 5 $\mu\text{g/ml}$ for 24 h is reached with a maximum blood concentration of $97.5 \pm 18.1 \mu\text{g/ml}$ in one hour (Figure 5C). The analysis of the pharmacokinetic variables of amoxicillin concentration changes in flatfish plasma after doses of 10, 20 and 40 mg/kg of amoxicillin in muscle are shown in Table 5. Standard curve was produced with solution ranging between 0.5 to 10 $\mu\text{g/ml}$ and R^2 was determined 0.9999. The recovery rate of amoxicillin was 106.6-120 %.

Figure 5. Amoxicillin plasma concentrations in flatfish after administration of an intramuscular injection of 10 mg/kg (A), 20 mg/kg (B) and 40 mg/kg (C) of amoxicillin. (Park *et.al.*, 2013)

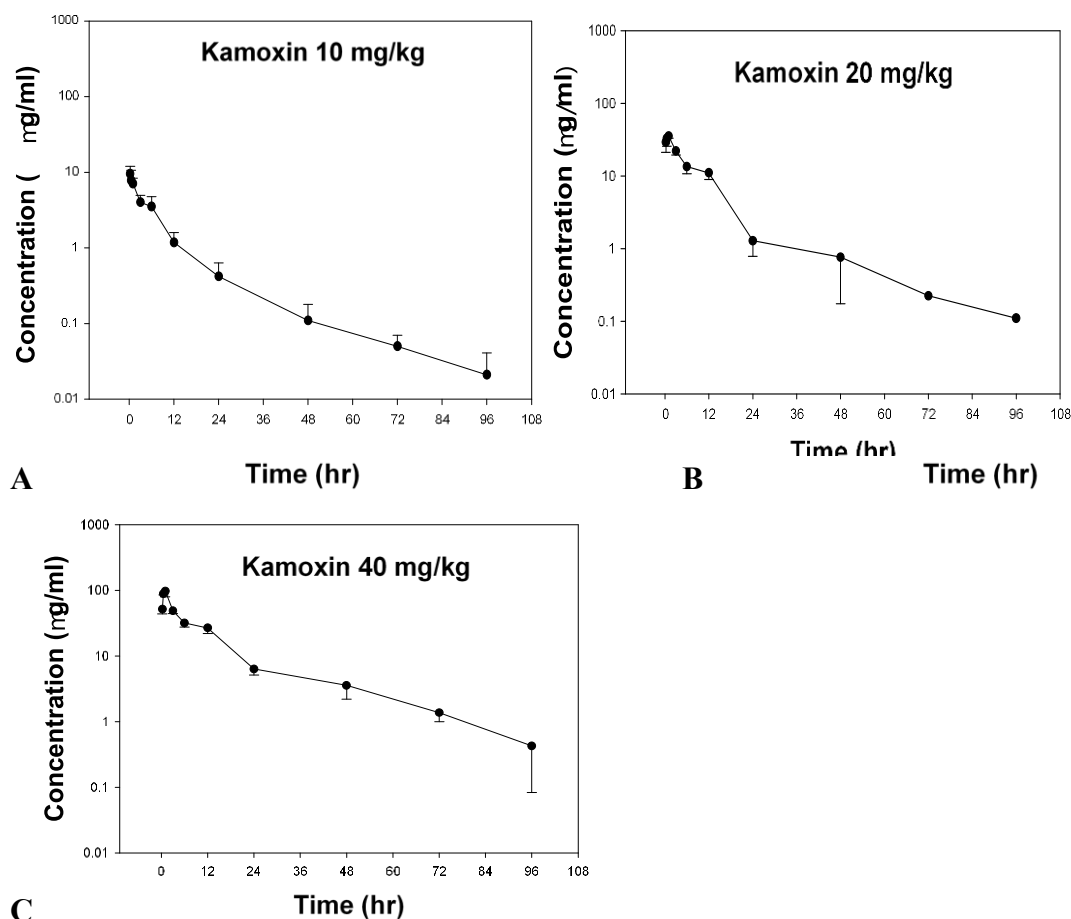


Table 5. Analysis of pharmacokinetic variables of amoxicillin concentration changes in flatfish plasma after amoxicillin intramuscular dose of 10 mg/kg, 20 mg/kg and 40 mg/kg

Parameter	Unit	Value		
		10 mg/kg	20 mg/kg	40 mg/kg
$t_{1/2}$	h	20.09	19.37	15.70
t_{max}	h	0.25	1	1
C_{max}	µg/ml	9.53	35.34	97.51
AUC	µg/ml h	62.24	325.8	912.4
MRT	h	12.06	12.43	16.41

$T_{1/2}$, Elimination half-life; t_{max} , The time of peak concentration; C_{max} , The peak or maximum concentration; AUC, Area under a concentration of analyte vs. time curve from zero to time infinity; MRT, Mean Residence Time

Sea bream

A study was conducted to investigate serum and tissue concentrations of amoxicillin in sea bream (*Sparus aurata* L.). Fishes (weight ranged from 120 to 160 g) were divided into two groups and acclimated for 7 days in tanks (water temperature of 22 °C and 32 ‰ of salinity). The first group was treated with a single intravenous (i.v.) dose of 40 mg/kg bw and the second group received amoxicillin in feed corresponding to a single oral dose of 80 mg/kg bw (Della Rocca, *et.al.*, 2004). To facilitate the intravenous injection and the collection of the blood, the fish were anesthetized. Blood was collected by puncturing the caudal vein from 10 fish at each of the following time points: just before drug administration (time 0) and then at intervals ranging from 0.5 to 72 h after i.v. injection and from 1 to 24 h after oral administration. Samples of tissues (muscle, liver and skin) were also collected at 0.5 (i.v. administration), 1, 3, 6, 8, 10, 12, 24 and 72 h. Radioimmunoassay-microbial receptor technology (Charm II test for β -lactams – LOD of 5 µg/L) and microbiological assay (spores of *Bacillus stearothermophilus* ATCC 10149– LOD of 2 µg/L) were used to determine the amoxicillin concentrations in pooled sera. Tissues (muscle, skin, liver) levels were obtained by Charm II test. Although some variability between the values obtained by the two methods was evident, no statistically significance ($p < 0.001$) was found. After intravenous administration, the pooled serum concentrations decreased very slowly. After i.v. treatment, the concentrations ranged from 0.94 to 0.66 µg/ml at 72 h. A maximum concentration of 1.00 µg/ml of amoxicillin in serum was reached 8 h after oral administration but decreasing to a level of 0.1 µg/ml at 16 h. The maximum concentration was found at the first hour after oral treatment in liver with 430 ± 250 µg/kg, and at the 6th hour in muscle and skin with concentrations of 190 ± 70 and 160 ± 80 µg/kg, respectively. A poor amoxicillin distribution and a sharply reduction of amoxicillin concentrations from muscle and skin, were observed in a single feeding dose treatment. The amoxicillin serum concentration–time course in treated seabream dosed by i.v. administration decreased very slowly, indicating a slow clearance from the excretory organs. The $AUC_{0-72\text{ h}}$ values of 2128 and 14 µg/ml × h obtained after i.v. and p.o. treatment, respectively, confirmed that the oral absorption was incomplete. The oral bioavailability (F %) was calculated to be 0.33 %. Although the sensitivity of the Charm II test was 5 µg/L, in the context of the analytical methods employed, the serum concentrations were found to be underestimated

by this test. Also, this test is not specifically aimed at measuring actual concentrations and is not specific to differentiate between antibiotics members of the same family.

Salmon

A field trial study was conducted by Brown and Grant 1992, to determine the pharmacokinetics of amoxicillin in Atlantic salmon (*Salmo salar*) after intramuscular injection. Sixty-seven animals (weight range of 3.3 kg to 4 kg) were farmed at water temperature ranging between 12 to 13 °C. along the study. They were slightly anaesthetized with benzocaine and treated with a single dose of 12.5 mg/kg of amoxicillin by a deep intramuscular injection in the caudal dorsal fin. Blood samples from 10 randomly selected fish were collected at time 1.5, 4, 24, 96, 144, 192, 288 and 336 h after treatment. Serum was analyzed for the presence of amoxicillin according to the reference Sutherland and Robinson (1978). Amoxicillin concentrations in serum after injection of standard 45 mg of amoxicillin trihydrate (to reach the dose of 12.5 mg/kg to medicate the average fish) at day 0 were measured. At approximately 24 h the amoxicillin concentration peaked, followed a sigmoidal pattern of excretion, dropping at day 12 below 125 µg/L. A standard amount of amoxicillin was giving to different sized fish, which could be the reason for the large standard deviation in the results.

Two different other studies were conducted to determine the efficacy of amoxicillin in relation to severity of the furunculosis challenge, and amoxicillin concentration levels in salmon plasma were investigated (Inglis *et.al.*, 1992). In one study, groups of 5 fish (average weight 11.5 g) were lightly anaesthetized to administrate by gastric intubation (gavage) a single dose of amoxicillin of either, 1200 µg, 480 µg, 240 µg, or buffer control. Blood samples were collected after 2 h of gavage and amoxicillin concentrations from the pooled plasma samples were determined by bioassay (LOD of 300 µg/L). In a different study, four groups of 10 fish were acclimatized to 18 °C and feed with amoxicillin medicated feed twice per day to deliver an oral dose of 80, 40 and 20 mg/kg bw/day, during 5 days. Blood samples were collected on the fifth day of treatment, one hour after the first feed. Amoxicillin concentrations from pooled plasma samples from 10 fish were determined by bioassay (Table 6).

Table 6. Amoxicillin concentrations obtained by bioassay, from pooled plasma samples of salmon treated by feed (dosed twice/day for 5 days, sampled 1 h after the 1st dose on the 5th day of treatment) or by gavage (single dose, sampled 2 h after gavage) (LOD = 0.3 µg/ml). (Inglis *et.al.*, 1992)

Dose of amoxicillin	Amoxicillin concentration in plasma (µg/ml)
80 mg/kg in diet	1.25 approximately
40 mg/kg in diet	0.3 - 0.6
20 mg/kg in diet	< 0.3
Normal diet	< 0.3
1200 µg/fish by gavage	1.25 – 2.5
480 µg/fish by gavage	0.3 - 0.6
240 µg/fish by gavage	< 0.3
Control	< 0.3

Another study was conducted to determine the amoxicillin serum concentrations reached in Atlantic salmon (*Salmo salar* L) parr after oral administration of 80 mg of amoxicillin/kg bw/day, delivered as medicated feed twice a day for three days (Inglis *et.al.*, 1993). Two groups of 20 healthy salmon (mean body weight, 12.5 g and range, 10.2-15.5 g) were acclimatized in 2 tanks with flow-through fresh water system (16 °C) until fish regular behavior was established. Blood samples from three fish were collected after the first feed on the fourth day, at 30 min and 1, 2, 3, 4, and 5 h after the morning feed. Samples from groups of three animals were pooled and amoxicillin concentrations were determined by bioassay with LOD of 0.3 µg/ml (same method as Inglis *et.al.*, 1992). After 30 minutes of administration, no amoxicillin could be detected; amoxicillin concentrations rapidly increased after 1 and 2 h reaching 0.6 and 0.8 µg/ml, respectively. After 2 h of administration the peak concentration occurred, decreasing to 0.5 µg/ml after three h post treatment and gradually decreasing more during the following two h.

Two comparative tables summarizing the information described above are included below (Table 7 and Table 8).

Table 7. Comparative description of important pharmacokinetic parameters in serum from olive flounder, flatfish and eel

Reference	Fish	Drug	Route of adminis- tration	Dose mg/kg bw/day	Days	C _{max} (µg/ml)	t _{max} (h)	AUC (µg/mL h)	
Seo <i>et.al.</i> , 2014	Olive flounder	amoxicillin trihydrate	Gavage	40	1	1.144	1.702	52.257	
				80	1	0.766	1.636	41.219	
				30	1	4.051	29.8	370.274	
				60	1	4.317	38.65	453.655	
Lim <i>et.al.</i> , 2016		amoxicillin sodium	i.m.	40	1	62.64	1.59	933.23*	
				80	1	87.61	3.02	1592.55*	
Park <i>et.al.</i> , 2016				12.5	1	22.05	2.6	273.69*	
				125	1	106.76	2.20	2755.37*	
Seo <i>et.al.</i> , 2014		Eel	amoxicillin trihydrate	i.v.	6	1	9.076	2.171	86.274
Jeon <i>et.al.</i> , 2010				Feed	40	1	3.04	2.1	464
					80	1	3.4	3.6	667
				i.v.	1	1	4.2	9	748
Park, <i>et.al.</i> , 2013	Flatfish			amoxicillin sodium	i.m.	10	1	9.5	0.25
		20	1			35.3	1	325.8	
		40	1			97.5	1	912.4	

*AUC_{0-inf}

Table 8. Comparative amoxicillin residue levels found in plasma of salmon, sea bream and pompano treated with amoxicillin trihydrate

Reference	Fish	Route of administration	Dose mg/kg bw/day	Days	Peak level (µg/ml)	Time when peak reached (h)
Brown and Grant 1992		i.m.	12.5	1	--	24
Inglis <i>et.al.</i> , 1992	Salmon	Gavage	1200 µg ¹	1	1.25 - 2.5 ²	2
			480 µg ¹	1	0.3 – 0.6 ²	2
			240 µg ¹	1	<0.3 ²	2
		Feed	80	5	1.25 ²	1
			40	5	0.3 – 0.6 ²	1
			20	5	<0.3 ²	1
Inglis <i>et.al.</i> , 1993			80	3	0.8 ²	2
Della Rocca <i>et.al.</i> , 2004	Sea bream	Feed	80	1	1 ³	8
		i.v.	40	1	--	--
Wang <i>et.al.</i> , 2015a	Pompano	Feed	40	1	7.36 µg/g	0.5
			40	5	--	--

-- value not reported in the manuscript, ¹ fix volume independently of the fish size, ²Tested by bioassay, ³Tested by microbiological assay and Charm II.

Tissue residue depletion studies

Radiolabelled residue depletion studies

No residue depletion studies using radiolabelled amoxicillin in cattle, pigs or sheep were provided for evaluation at the 75th meeting of the Committee.

No residue depletion studies in fish using radiolabelled amoxicillin were provided to the present Committee.

Residue depletion studies with non-radiolabelled drug

Fish

Twelve studies investigating the residue depletion of amoxicillin in fish tissues were submitted for evaluation or have been found available in the public domain. The studies include ten different types of fish: flatfish (Park *et.al.*, 2013), grouper (Wang *et.al.*, 2009), bass (Kuo *et.al.*, 2009), catfish (Ang *et.al.*, 2000), salmon (Inglis *et.al.*, 1992), pompano (Wang *et.al.*, 2015b), sea bream (Della Rocca, *et.al.*, 2004), rockfish and sea bream (Chung *et.al.*, 2006; Park *et.al.*, 2010); and olive flounder, (Chung *et.al.*, 2006; Park *et.al.*, 2010; Son *et.al.*, 2011; Park *et.al.*,

2016; Lim *et.al.* 2016). None of these studies are reported as GLP studies. They are briefly described below and are listed according to the fish species tested in each study.

Olive flounder

A study was performed to investigate amoxicillin residues in muscle of olive flounder (*Paralichthys olivaceus*) after oral administration of 40 mg/kg bw/day or 80mg/kg bw/day of amoxicillin (Son *et.al.*, 2011). Five hundred healthy olive flounders (550 ± 150 g, 34 ± 5 cm) with no history of antibiotic use, were located into a concrete tank to conduct a 30 days study over two trials: (1), an intake of 40 mg of amoxicillin per kg bw per day, at water temperature of 13.4-15.6 °C and (2), an amoxicillin dose of 80 mg/kg bw/day at water temperature of 16.8-21.4 °C. Animals were treated for 7 consecutive days. After treatment, ten muscle samples in each time point were collected until reached a residue level below 50 µg/kg. Amoxicillin concentrations in muscle were determined by a HPLC method. The amoxicillin LOD was 2.1 µg/kg and the LOQ was 7 µg/kg. The amoxicillin recovery rate from olive flounder muscle samples resulted in an average of 103.8 % and 100.8 % when added 500 and 1000 µg/kg of amoxicillin, respectively.

Under the examination condition (1) - lower temperature and dose of 40 mg/kg bw/day - the maximum accumulation of amoxicillin in olive flounder muscle during treatment was found on day 5 with an average of 303 ± 73 µg/kg. One day after the last administration, the average amoxicillin residue level in muscle was 188.5 ± 159.7 µg/kg, decreasing to 25.0 ± 33.4 µg/kg by day 10. By day 20, the amoxicillin concentrations were lower than the LOQ of 7 µg/kg. Under the examination conditions (2) – higher temperature and dose of 80 mg/kg bw/day - the maximum average concentration of amoxicillin in olive flounder muscle was found on day 5 of treatment with an average of 624 ± 323 µg/kg of amoxicillin. One day after the last administration, the mean amoxicillin residue level in muscle was 89 ± 33.6 µg/kg, but decreasing to 20.0 ± 7.7 µg/kg by day 5 (Table 9). The amoxicillin concentrations were lower than the LOQ of 7 µg/kg by day 13. Raw data were sent by the authors; the amoxicillin concentrations in olive flounder muscle samples after oral administration data was analyzed (Figure 6, Figure 7).

Table 9. Amoxicillin concentrations (Mean \pm SD) in muscle of olive flounder during and after treatment with either 40 mg/kg bw/day or 80 mg/kg bw during seven days. (Song *et.al.*, 2011)

Treatment	Sample time (days)	Amoxicillin muscle concentrations (µg/kg)			
		N > LOQ	Animals dosed with 40 mg/kg bw at 13.4-15.6 °C	N > LOQ	Animals dosed with 80 mg/kg bw at 16.8-21.4 °C
During	1	9	150.8 ± 122.5	8	269.7 ± 88.0
	3	10	131.8 ± 143.2	10	252.1 ± 159.3
	5	10	302.6 ± 173.1	10	624.4 ± 323.4
	7	10	118.2 ± 92.4	10	316.2 ± 69.3
Post	1	10	188.5 ± 159.7	10	89.0 ± 33.6
	3	10	93.7 ± 50.2	10	51.4 ± 12.2
	5	9	56.2 ± 23.8	8	20.0 ± 7.7

7	8	58.0 ± 14.1		NS
9	10	61.6 ± 21.2	7	9.6 ± 1.6
10	9	25.0 ± 33.4	2	7.5 ± 0.7
13	7	18.3 ± 13.9	0	--
20	0	--	0	--

N>LOQ : Number of fish samples with concentrations below the LOQ, NS : Not sampled

Figure 6. Amoxicillin concentrations in olive flounder muscle samples after oral administration of amoxicillin at 40 mg/kg/bw/d for 7 days.

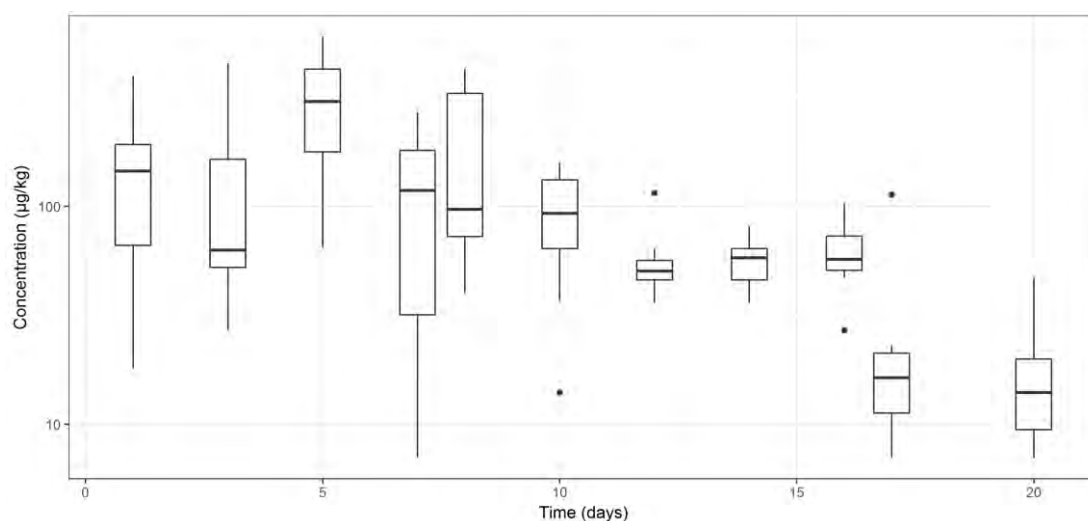
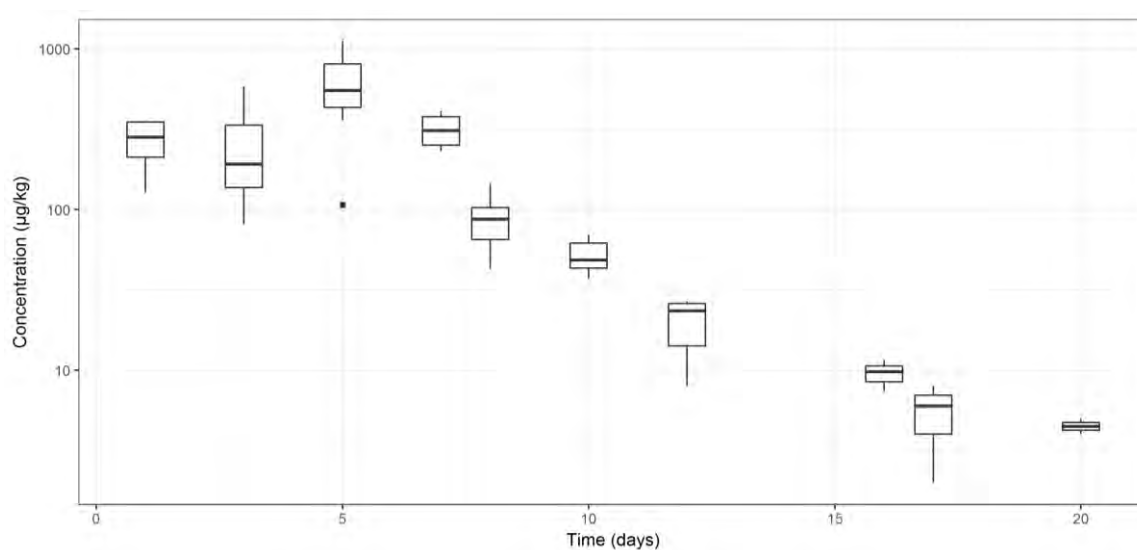


Figure 7. Amoxicillin concentrations in olive flounder muscle samples after oral administration of amoxicillin at 80 mg/kg/bw/d for 7 days



Comparison of both studies shows that even at higher dose of amoxicillin treatment, the increase of the temperature, results in faster depletion of the residues to levels <7 µg/kg (LOQ)

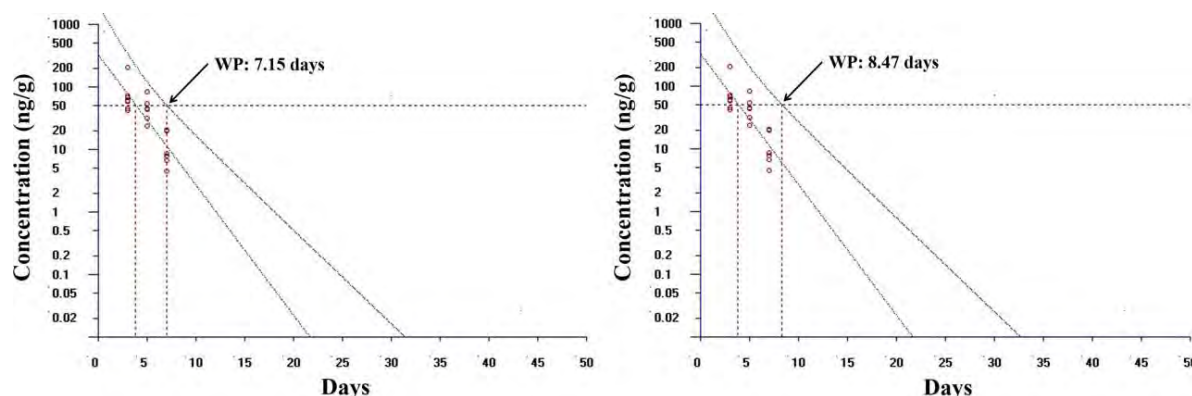
(it takes almost half of the time to reach the same low level residue concentration at higher temperatures).

A study was conducted to determine amoxicillin tissue concentrations in samples from olive flounder (*Paralichthys olivaceus*) treated with a single intramuscular dose of either 40 or 400 mg/kg of amoxicillin sodium (Lim *et.al.* 2016). Ten healthy olive flounder (average weight of 87 ± 16 g) per tank were acclimatized for one week in filtered seawater maintained at $22 \text{ }^{\circ}\text{C} \pm 1 \text{ }^{\circ}\text{C}$. Muscle samples were collected from 10 fish per time point at 3, 5, 7, 12, 15, 17, and 21 days after dose. Animals were anesthetized before sampling. Amoxicillin concentrations in muscle samples (skinless) were determined using a validated LC-MS/MS method and extraction procedure outlined by the Korean Food Standards Codes (MFDS, 2012) with some modifications. The LOD and LOQ were calculated to be 2.36 and 7.16 $\mu\text{g/L}$, respectively. Amoxicillin average recoveries from muscle, determined by LC-MS, after doses of 0.01 and 0.1 $\mu\text{g/ml}$ were 98.0 and 111.0 %, respectively. Amoxicillin muscle concentrations after treatment with a single i.m. dose of either 40 or 400 mg/kg bw are shown in Table 10. Amoxicillin concentrations in muscle of olive flounder treated with a single i.m. single dose of 40 mg/kg bw, decreased rapidly from $71.2 \pm 54.9 \text{ } \mu\text{g/kg}$ (at day 3 post injection) to $11.3 \pm 6.4 \text{ } \mu\text{g/kg}$ after 7 days from treatment, and reaching undetectable levels at 12 days post injection. However, amoxicillin concentrations in muscle of olive flounder treated with a higher single i.m. dose of 400 mg/kg bw, showed lower rate of depletion, decreasing from $1975.9 \pm 639 \text{ } \mu\text{g/kg}$ (at day 3 post injection) to $14.2 \pm 3.5 \text{ } \mu\text{g/kg}$ after 12 days from treatment and reaching undetectable levels at 21 days post injection. The calculation of withdrawal period is shown in Figure 8. The statistical analysis for the withdrawal period was done according CVMP and EMEA, 1996 guidelines, considering an MRL of 50 $\mu\text{g/kg}$ in edible fish. Withdrawal period was determined as the time at which the 95 or 99 % upper one side tolerance limit was below the MRL with 95 % confidence, 7.15 days or 8.47 days after dose of 40 mg/kg bw fish.

Table 10. Amoxicillin muscle concentrations after olive flounder treatment with a single i.m. dose of either 40 or 400 mg/kg bw (Lim *et.al.*, 2016)

Days Post- injection	Amoxicillin muscle concentrations ($\mu\text{g/kg}$)	
	Animals dosed with 40 mg/kg	Animals dosed with 400 mg/kg
3	71.2 ± 54.9	1975.9 ± 639.0
5	55.1 ± 18.1	-
7	11.3 ± 6.4	1213.4 ± 199.7
12	Not detected	14.2 ± 3.5
21	-	Not detected

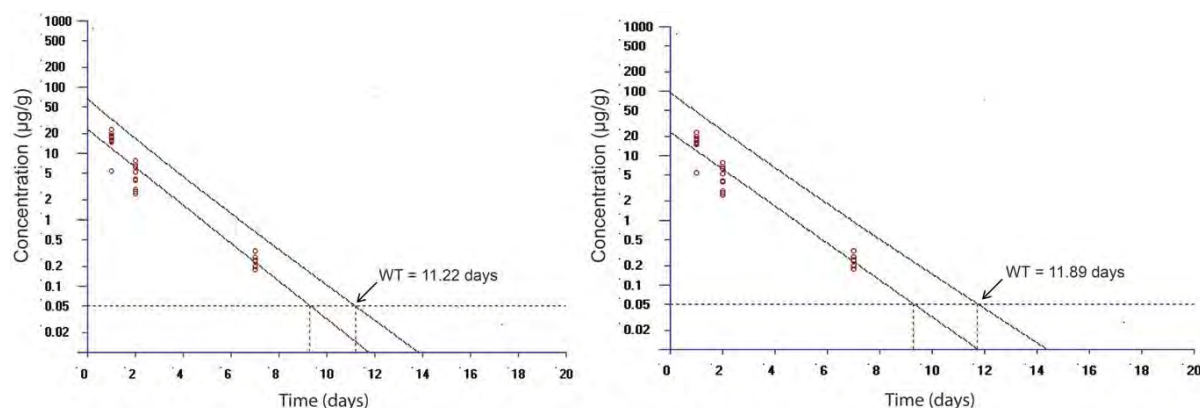
Figure 8. Amoxicillin residue depletion graphs from muscle of olive flounder treated with 40 mg/kg bw (95 % (left) and 99 % (right) statistical tolerance limit with 95 % confidence). (Lim *et.al.*, 2016)



A withdrawal period was estimated as the time at which the 95 or 99 % upper one side tolerance limit was below the proposed MRL of 50 µg/kg with 95 % confidence, 7.15 days or 8.47 days after dose of 40 mg/kg bw fish. No reference of water temperature was included in this calculation.

A tissue depletion study was conducted to estimate the amoxicillin concentration in tissues of olive flounder (*Paralichthys olivaceus*) dosed with a daily amoxicillin sodium injection of 37.5 mg/kg continuously for 1 week (Park *et.al.*, 2016). Four groups of 10 fish (average weight of 140 ± 22 g and age ranged from 150 to 180 days) were acclimatized for 2 weeks in fresh water at 23 ± 1 °C. After the last administration, combined muscle plus skin fish samples were collected at 1, 2, 7 and 14 days post treatment. Amoxicillin concentrations in muscle plus skin samples were determined using a validated HPLC method. The amoxicillin average tissue concentrations were 17100, 4700, and 300 µg/kg at 1, 2, and 7 days, respectively, after the last administrated dose of 37.5 mg/kg. At day 14, most of concentration data was below the LOQ of 40 µg/kg. Figure 9 shows the amoxicillin depletion studies from muscle plus skin of treated olive flounder. Withdrawal period estimation was done according to the European Medicines Agency (EMA, 2002) and U.S. Food and Drug Administration (2006) guidelines, considering the MRL of 50 µg/kg in edible tissues (EMA, 2008). It was determined as the time when the 95 % or 99 % upper one side tolerance limit was below the MRL with 95 % confidence, 11.2 d or 11.8 days, respectively, which was rounded to 12 days. Since fish were kept in mean daily temperature of 23 °C, then the withdrawal time is calculated as 276 degree-days.

Figure 9. Amoxicillin depletion data from muscle plus skin of olive flounder (n=40) after the last intramuscular administration of 37.5 mg/kg amoxicillin sodium for 1 week. Withdrawal time (WT) in this graph was calculated considering the European MRL of 50 µg/kg in tissues and indicates the time when the linear regression lines of upper one-sided tolerance limits at 95 % (left panel) and 99 % (right panel) with 95 % confidence fell below the MRL for amoxicillin in fish muscle and skin. (Park. *et.al.*, 2016)



Based on an MRL established of 50 µg/kg for amoxicillin in fish tissues, a withdrawal period for amoxicillin sodium from muscle plus skin of 12 days, at water temperature of 23 °C (276 degree-days), is proposed.

Olive Flounder - Rockfish - Red Sea Bream

A study was performed by Chung *et.al.*, (2006) to examine the amoxicillin residue distribution in muscular tissues of olive flounder (*Paralichthys olivaceus*), rockfish (*Sebastes schlegeli*), and red sea bream (*Pagrus major*). The fish average weight was 360 ± 40 g, 470 ± 55 g, and 450 ± 45 g, respectively. Each fish species group included 25 animals. They were acclimatized for 15 days in a recirculating water tank with controlled temperature of 25 °C. Amoxicillin was administered orally with medicated feed at a dose of 400 mg/kg bw during 7 days. Five animals were slaughtered the 1st, 2nd, 3rd, 4th, and 5th day after the last amoxicillin administration and muscle samples were collected. A validated method using sample clean-up and high performance liquid chromatography with fluorescence detector was performed to determine the amoxicillin concentrations in muscle samples. The mean recovery was found to be 97.3 and 86.7 % (olive flounder), 95.7 and 82.9 % (rock fish) and 96.5 and 87.8 % (red sea bream), when muscle samples were spiked with 50 and 100 µg/kg of amoxicillin, respectively. One day after the last administration, the mean amoxicillin concentrations detected in the muscle were 137 µg/kg in olive flounder, 131 µg/kg in rockfish, and 172 µg/kg in red sea bream. Mean concentrations were reduced after 3 days of treatment to 12, 10, and 17 µg/kg, respectively. By the fourth day after treatment, all fish samples were below the LOD of 10 µg/kg. The amoxicillin concentrations (mean and standard deviations) detected in fish muscle samples are shown in Table 11.

Table 11. Amoxicillin concentrations obtained from fish muscle samples after treatment with 400 mg/kg bw/day of amoxicillin in feed for 7 days. (Chung *et.al.*, 2006)

Fish	N° of samples	Amoxicillin concentration (µg/kg) after treatment			
		1 day	2 day	3 day	4 day
<i>P. olivaceus</i>	5	137 ± 39	42 ± 25	12 ± 8	ND
<i>S. schlegeli</i>	5	131 ± 43	38 ± 17	10 ± 9	ND
<i>P. major</i>	5	172 ± 53	53 ± 29	17 ± 6	ND

ND: Not detected

A different study was conducted to demonstrate the application of a solid-phase fluorescence immunoassay for analysis of amoxicillin in fish tissue (Park *et.al.*, 2010). Three groups of fish, 25 olive flounder (*Paralichthys olivaceus*), 25 rockfish (*Sebastes schlegeli*) and 25 red sea bream (*Pagrus major*), were orally dosed with amoxicillin trihydrate 400 mg/kg bw/day for 7 consecutive days. The fish average weight was 350 ± 30 g, 450 ± 40 g, and 420 ± 35 g, respectively. Each fish species group included 25 animals. They were acclimatized for two weeks in indoor circular tanks with controlled temperature of 25 ± 1 °C. Muscle samples were collected from five fish of each group, on day 1, 2, 3, 4 and 5 after amoxicillin treatment. Samples were coded for blind analysis. Amoxicillin concentrations in muscle were determined using a solid-phase fluorescence immunoassay based test, designed for milk analysis. The LOD was calculated to be less than 10 µg/kg. The recovery rate was found to be 91.5 and 86.6 % (olive flounder), 90.0 and 88.7 % (rock fish) and 90.7 and 85.9 % (red sea bream), when muscle samples were spiked with 10 and 50 µg/kg of amoxicillin, respectively. Table 12 shows the amoxicillin residue levels found in muscles samples from olive flounder, rockfish and red sea bream after treatment. Amoxicillin residues levels decreased below 10 µg/kg, in all muscle tissue samples, after 5 days from treatment.

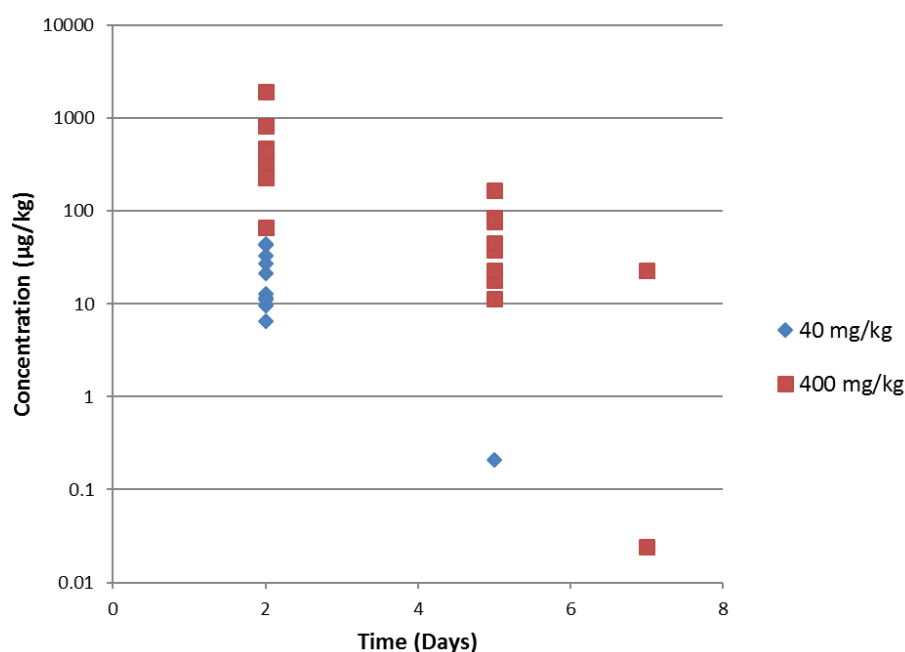
Table 12. Amoxicillin residue levels in muscle samples from animals orally dosed with amoxicillin 400 mg/kg bw for 7 consecutive days. (Park *et.al.*, 2010)

Fish	Withdrawal (days)	N° of positive results	Amoxicillin concentration (µg/kg)
<i>P. olivaceus</i>	1	5	84.3 ± 2.1
	2	5	55.2 ± 1.1
	3	5	12.6 ± 0.5
	4	0	<LOQ
<i>S. schlegeli</i>	1	5	84.1 ± 2.0
	2	5	33.9 ± 1.0
	3	4	10.0 ± 0.4
	4	0	<LOQ
<i>P. major</i>	1	5	96.2 ± 2.3
	2	5	53.8 ± 1.4
	3	5	13.6 ± 0.5
	4	0	<LOQ

Flatfish

A study was conducted to determine the amoxicillin concentration in flatfish edible tissues after administration of an amoxicillin sodium dose of either 40 mg/kg or 400 mg/kg (Park *et.al.*, 2013). After over one week of domestication in culture water with salinity of 28 psu and at 22 ± 3 °C, depending of the groups, 40 mg/kg or 400 mg/kg of amoxicillin were administrated intramuscularly in flatfish (weight 150 ± 20 g). Ten muscle samples were analyzed quantitatively after 2, 5, 7, 14, 21, and 30 of administration using LC-MS/MS. The LOD of amoxicillin was reported as 0.005 µg/kg and no LOQ was provided. Results are shown in Figure 10. After two days of the administration, all amoxicillin concentrations found in flatfish muscle samples from animals treated with 40 mg/ml were below 50 µg/kg and the mean amoxicillin concentration in muscle was 23.3 ± 14.1 µg/kg. Only one of the ten fish sampled at 5 days had a detectable amoxicillin residue (0.21 µg/kg). Ten samples exceeded the 50 µg/kg concentration after 2 days of administration of 400 mg/kg. Three out of the 10 samples were still above 50 µg/kg after 5 days of amoxicillin administration. However, none of the samples from animal dosed with either one of the amoxicillin concentrations exceed 50 µg/kg after 7 days of administration.

Figure 10. Amoxicillin concentrations found in flatfish muscle samples from animals treated with either 40 mg/kg or 400 mg/kg.



In a different study, seven flatfish muscle samples randomly collected from a field clinical trial at two aquafarms, were analyzed to determine the amoxicillin concentration in these tissues on days 5, 7 and 14 after treatment with 40 mg/kg of amoxicillin (Park, *et.al.*, 2013). Results show that none of the samples exceed the reported LOD concentration of 0.005 g/kg, after 5 days of administration.

Pompano

In two scientific papers (Wang *et.al.*, 2015a, Wang *et.al.*, 2015b), a comparison of amoxicillin depletion curves in serum and tissues was performed in pompano (*Trachinotus blochii*) after single or repeated oral doses (5 days) of amoxicillin trihydrate (40 mg/kg bw). Fish (mean bw of 160.4 ± 27.7 g) were maintained in pools with aerated seawater at temperatures ranging from 25 °C to 27 °C, 8-10 ppm of oxygen content and 3.3 ‰ of salinity. Dose was delivered by placing a syringe in the fish mouth through the esophagus into the stomach. After oral gavage, seven fish treated with a single dose of 40 mg/g bw/day were sacrificed and tissues as well as blood samples were collected at 0.5, 1, 2, 4, 6, 12, 24, 48, 72, 96, 120, 144 and 168 h after treatment was finalized to determine the amoxicillin residue concentrations. Samples were stored at -80 °C. In the study where the fish were dosed with 40 mg/kg/day for five consecutive days, the samples were collected at 0.5, 1, 2, 3, 4, 5, 6 and 8 days after the last treatment. Samples were stored at -20 °C. Serum, muscle, liver and kidney concentrations were determined by HPLC-UV (LOD of 40 µg/kg, LOQ of 100 µg/kg). A recovery range of 74.3 % to 83.4 % was obtained when muscle tissue samples were spiked with 0.05, 0.1, 0.5, and 1 µg/g of amoxicillin. The coefficients of variation were less than 10 %. The amoxicillin concentrations in the serum and muscle tissue samples after pompano treatment with either a single oral dose or administrations for five consecutive days of a 40 mg/ml dose are represented (See figure 2 and 3, respectively, on Wang *et.al.*, 2015a). After 30 min of a single oral amoxicillin dose of 40 mg/kg, the maximum concentration in serum was reached at 7360 µg/L, meanwhile, the maximum concentration of amoxicillin in muscle (1960 µg/kg) was reached after 2 h post treatment. The amoxicillin concentrations declined with half-lives of 7.4 and 17.3 h, in serum and muscle samples, respectively. Serum samples from animals treated with five constitutive daily doses of 40 mg/ kg of bw, showed amoxicillin residues decreasing to undetectable at day 2, while that was also the case in muscle samples, but at day 1 after administration. The amoxicillin concentrations decreased lower than LOD in serum and muscle after 48 h and 72 h, respectively. After 30 minutes of a single amoxicillin oral dose of 40 mg/kg, maximum concentrations of amoxicillin, of 6170 µg/kg in liver and 4270 µg/kg in kidney, were reached. Subsequently, they declined with half-lives of 18.3 and 12.0 h, respectively. At day 3, amoxicillin residues in all tissues were non-detectable (Figure 11). In the group of fish receiving a dose of 40 mg/kg/day for five consecutive days, the highest amoxicillin concentrations in liver and kidney tissue were determined on day 0.5 at 530 and 860 µg/kg, respectively, declining in the following days. Amoxicillin residues declined to undetectable levels on day 2 for liver samples and on day 3 for kidney samples (Figure 12). Therefore, peak concentrations in serum, liver and kidney (7360 µg/L, 6170 and 4270 µg/kg) were reached 0.5 h post dosing after a single administration, while the peak concentration in muscle (1960 µg/kg) was observed 2 h post-dosing. The concentrations of amoxicillin in serum, liver, kidney and muscle declined with half-lives of 7.4, 18.3, 12 and 17.3 h respectively.

Figure 11. Amoxicillin residues in liver and kidney of pompano after treatment with a single oral dose of 40 mg/kg bw. (Wang *et.al.*, 2015b)

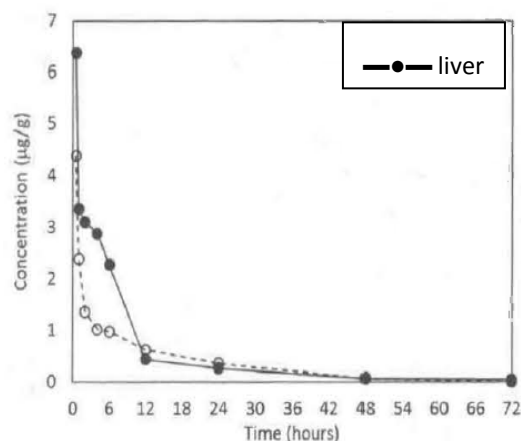
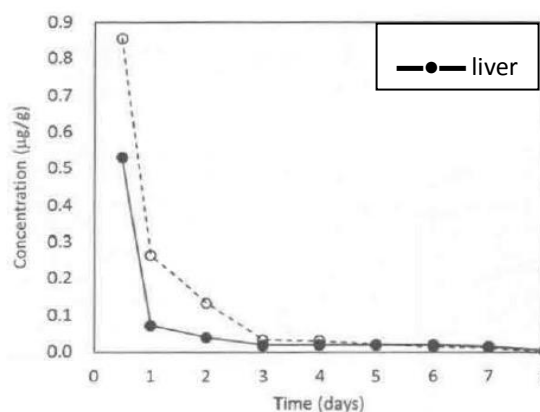


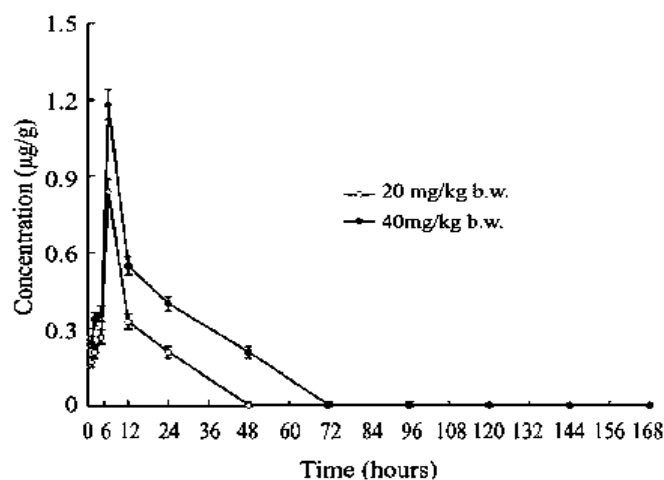
Figure 12. Amoxicillin residues in liver and kidney pompano after treatment for five consecutive days at a daily dose of 40 mg/ml of amoxicillin (Wang *et.al.*, 2015b)



Bass

A study was conducted by Kuo *et.al.*, 2009 to determine the amoxicillin residue concentration in muscle of bass (*Micropeterus salmoides*) after oral administrations of amoxicillin trihydrate. Largemouth black bass (weigh of 230 ± 10 g) were acclimatized in fresh water tank for one week (18 ± 0.8 °C). Amoxicillin was administered as the dosage 20 or 40 mg/kg bw/day for 5 consecutive days. Muscle tissue samples from six fish were collected after the last administration at 1, 2, 4, 6, 12, 24, 48, 72, 96, 120, 144, 168, 192 h until 10 days and stored at -80 °C until analyzed. Amoxicillin levels in muscle were determined using a solid-phase extraction and high performance liquid chromatography (HPLC) method with UV detector. Accuracy, precision and linearity were determined intra-day and inter-day. Mean absolute recoveries were 78 %. The LOD was calculated as 40 µg/kg and the LOQ was 100 µg/kg. Muscle tissue samples from six fish were collected after treatment was finalized at 1, 2, 4, 6, 12, 24, 48, 72, 96, 120, 144, 168, 192 h until 10 days and stored at -80 °C until analyzed. After the completion of the 5 consecutive daily oral doses of amoxicillin, the maximum residues concentration (C_{max}) of 840 µg/kg or 1180 µg/kg were reached at 6-h-postdosing (t_{max}) for doses of 20 or 40 mg/kg bw/day, respectively. The average amoxicillin residue concentrations decreased to 210 µg/kg after 12-h of treatment with 20 mg/kg bw/day and 210 µg/kg after 24 h post treatment with 40 mg/kg bw/day of amoxicillin. Amoxicillin residues in bass tissue depleted a rapid rate during the first 24-h after fish were dosed with amoxicillin at 40 mg/kg bw/day during 5 consecutive days. After longer periods of depletion the variations ranges were narrower for bass. After 48 h post dose of 20 mg/kg bw dosage, residue concentrations in bass decreased to below the LOQ, with one exception at 100 µg/kg. Residue concentrations decreased to below the methods LOQ 72 h after the last oral treatment from all conditions, (Figure 13).

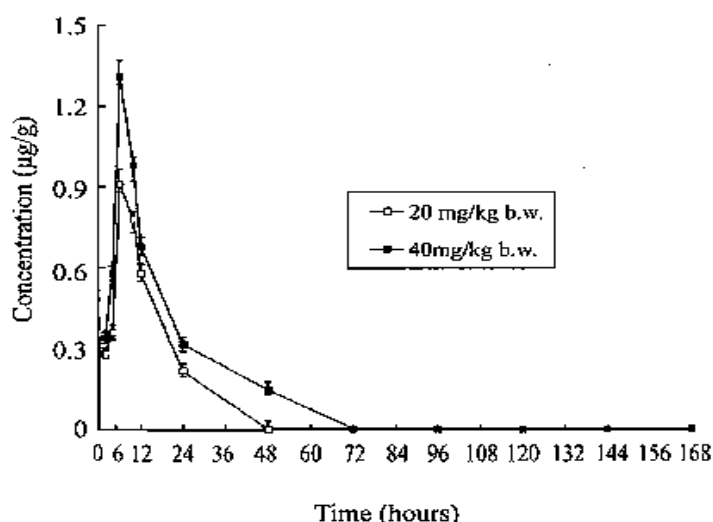
Figure 13. Amoxicillin muscle concentrations-time course curves for bass fish (mean \pm SD, n= 6) dosed with either 20 or 40 mg/kg bw/day for five consecutive days. (Kuo *et.al.*, 2009)



Grouper

A study was conducted to determine the amoxicillin concentrations in grouper (*Epinephelus malabaricus*) muscle after oral treatment, (Wang *et.al.*, 2009). Grouper fish (mean weight of 285 ± 15 g) were acclimatized in fresh water tank for one week. Water temperature was 18 ± 0.8 °C. Amoxicillin trihydrate was administered orally via medicated feed with either 20 or 40 mg/kg bw/day for five consecutive days. Muscle tissue samples from five fish were collected after treatment was finalized at 2, 4, 6, 10, 12, 24, 48, 72, 96, 120, 144 h, until 168 h and stored at -80 °C until analyzed. Amoxicillin concentrations in muscle were determined using an HPLC method with solid phase extraction and UV detection. Analytical performances of the HPLC method (linearity, recovery, precision, LOD and LOQ) were determined and the method was validated. The LOD was 40 µg/kg and the LOQ was 100 µg/kg. Amoxicillin average recovery was calculated to be 89.7 %. Amoxicillin concentrations reached the maximum level (C_{max}) of 910 and 1310 µg/kg at 6-h (t_{max}) after last dose of treatment with 20 and 40 mg/kg bw/day, respectively. The average amoxicillin residue concentrations decreased to 220 µg/kg after 24 h of treatment with 20 mg/kg bw/day and 150 µg/kg after 48 h post treatment with 40 mg/kg bw/day of amoxicillin. Amoxicillin residue concentrations in grouper muscle decreased below the LOQ after 48 h of fish treatment with 20 mg/kg bw/day for five consecutive days. Amoxicillin residue concentrations decreased to below the method LOQ at 72 h after the last oral treatment from all conditions (Figure 14).

Figure 14. Amoxicillin muscle concentrations-time course curves for grouper fish (mean \pm SD, $n=5$) dosed with either 20 or 40 mg/kg bw/day for five consecutive days. (Wang *et.al.*, 2009)



Catfish

A study was conducted to determine the incurred amoxicillin residue levels in catfish (*Ictalurus punctatus*) tissues following oral administration (Ang *et.al.*, 2000). Fish (0.5 to 1 kg of weight) were acclimatized for one week in indoor tanks (water temperature 27.5 ± 1.4 °C) before amoxicillin treatment. An oral dose of 110 mg of amoxicillin trihydrate per kg of fish was administered to anaesthetized catfish by gavage using a plastic pipette. Five fish were collected at each time interval of 6, 24, 48, and 72 h after treatment and samples were stored at -70 °C until analysis. Muscle fillets were analyzed using a HPLC method with precolumn derivatization and fluorescence detection.

Table 13 shows the amoxicillin concentrations of individual animals after oral administration at different depletion times. No interference was found in the analysis of these samples. The LOQ was 1.2 µg/kg. The amoxicillin concentrations varied between individual animals in the same group however, these variations were narrower after longer periods of depletion. 6 h after administration the range of amoxicillin concentrations in muscle was 40 to 64.2 µg/kg, except for one fish with a mean of 297 µg/kg. The average amoxicillin residue levels decreased to 5.4 and 2.9 µg/kg after 24 and 48 h of treatment, respectively. Depletion rate was fastest from 6 to 24 h post-treatment, under the conditions of this study. Although trace amounts of residues were still found after 72 h of depletion, the concentrations were below the LOQ. Depletion of amoxicillin residues in catfish muscle receiving 100 mg/kg treatment, is a fairly rapid rate retaining <10 µg/kg of the antibiotic after 24 h. After 72 h, the amoxicillin residues decrease to below the LOQ (<1.2 µg/kg).

Table 13. Amoxicillin concentrations found in catfish muscle samples after oral treatment of 100 mg/kg bw. (Ang *et.al.*, 2000)

Depletion time (h)	Fish	Fish Weight (kg)	Amoxicillin (µg/kg)	
			mean	SD
6	1	0.76	64.2	6.86
	2	0.56	50.6	3.82
	3	0.38	60.5	4.23
	4	0.48	40.0	7.17
	5	0.66	297	40.1
24	6	0.38	<LOQ	--
	7	0.36	7.3	0.51
	8	0.32	3.7	0.69
	9	0.44	7.0	0.12
	10	0.52	7.9	0.71
48	11	0.5	<LOQ	
	12	0.46	1.4	0.12
	13	0.54	6.9	0.21
	14	0.70	2.8	0.21
	15	0.38	1.9	0.2
72	16	0.48	<LOQ	--
	17	0.3	<LOQ	--
	18	0.44	<LOQ	--
	19	0.36	<LOQ	--
	20	0.36	<LOQ	--

LOQ=1.2 µg/kg

Sea bream

In addition to the study described earlier, Della Rocca and collaborators conducted a depletion kinetic study to determine the tissue concentrations of amoxicillin in sea bream (*Sparus aurata* L.) treated with medicated feed (using automatic feeders) during 10 consecutive days at the dose of 80 mg of amoxicillin per kg bw (Della Rocca, *et.al.*, 2004). One hundred ten seabream, weight of 50 to 80 g, were acclimatized for 14 days at water temperature of 22-26 °C and 32 ‰ of salinity. Tissues samples from muscle, skin, vertebrae and liver were collected from ten fish at each of the scheduled time points as follows: before treatment (time 0) and at 1, 5 and 10 days during treatment. After treatment was concluded, tissue samples were also taken every day for 5 days. Table 14 lists the mean amoxicillin concentrations in muscle, skin and liver samples of treated seabream determined by Charm II test with a limit of detection of 5 µg/L. The amoxicillin concentrations obtained from the seabream tissues examined during the treatment period were very low (13–35 µg/kg). After finalized treatment, a few scattered data of amoxicillin concentrations were measured in liver, skin and vertebrae. Immediately after the end of the 10-day treatment, the amoxicillin concentrations in muscle declined below the LOD,

but the mean levels in skin and liver resulted to be 28 ± 29 µg/kg and 11 ± 12 µg/kg, respectively.

Table 14. Amoxicillin concentrations (mean + S.D.) in tissues samples from seabream treated with 80 mg/kg bw/day for 10 days. (Della Rocca *et.al.*, 2004)

Treatment	Time (days)	Concentrations (µg/kg)			
		Muscle	Liver	Skin	Vertebrae
During	1	23 ± 11	< 5	20 ± 5	35 ^a
	5	13 ± 10	< 5	116 ± 69	6 ± 4
	10	< 5	11 ± 12	28 ± 29	< 5
Post	1	< 5	< 5	< 5	< 5
	2	< 5	66 ^a	< 5	< 5
	3	< 5	20 ^a	< 5	< 5
	4	< 5	< 5	13 ^a	20 ^a
	5	< 5	< 5	< 5	< 5

^a One positive sample.

Salmon

A study was conducted to determine the efficacy of amoxicillin in relation to severity of the furunculosis challenge, and amoxicillin concentration levels in salmon tissues were investigated (Inglis *et.al.*, 1992). Four groups of 20 fish were acclimatized to 18 °C; two groups received 5 oral daily doses of 80 mg/kg bw of amoxicillin and the other two received regular non-medicated feed. Two groups of fishes were sacrificed, 1 h after the last treatment and 12 days after, respectively. Tissues samples were collected and amoxicillin residue levels analyzed using the radiobiological assay Charm 7000 system. The LOD was found to be 5 µg/kg. The maximum level of drug was detected in the 1 h post treatment sample at > 0.32 µg/ml. The corresponding plasma level determined by bioassay was 1260 µg/L (or kg). After 12 days post treatment, amoxicillin concentrations were below the LOD of 5 µg/L (or kg).

For intramuscular administration of amoxicillin, no information on the position of sampling sites in relation to injection sites was provided in the residue depletion studies. However, considering the fish anatomy, the high solubility of amoxicillin sodium and the rapid absorption rate, the Committee considered it likely that concentrations at injection sites are similar to those in other muscle tissues

Monitoring data in several fish species

A study monitoring the occurrence of amoxicillin and ampicillin residues in fishery products in South Korea conducted from July 2014 to October 2015, is described (Anonymous, 2015). In this investigation, a total of 958 saltwater and freshwater fish samples [Halibut (51), Flatfish

(85), Sea bass (10), Convict grouper (5), Sea bream (73), Korean bullhead (28), Catfish (70), Loach (83), Croaker (3), Lobster (1), Rockfish (88), Crucian carp (44), Shrimp (119), Trout (8), Gray mullet (8), Salmon (82), Carp (35), Eel (123), Gizzard shad (33), Leather carp (9)] were collected from Korean retail stores and wholesale markets. After 4 h of transportation, minced edible tissues were stored at -20 °C. Samples were analyzed with an LC-MS/MS simultaneous multi-class detection method complying with the Korean Food Standard Code (MFDS, 2014). The parameters used for amoxicillin quantification are display in Table 15. Following CODEX guidelines (CAC/GL-16 and CL-71), validation was completed to evaluate the performance of the employed analytical method. With a LOQ of 5 µg/kg, recovery ranges of 61 -115 % and 60 – 104 % were obtained for flatfish and eel, respectively. The recovery and coefficients of variation for flatfish and eel are listed in Table 16. The results show a total amoxicillin detection rate of 1.25 % (12/958 = 1.3 %). Ten of the detected samples were olive flounder and the other two were salmon and shrimp, with an amoxicillin concentration range of 1-829 µg/kg and a mean of 115 µg/kg. Three of the flatfish samples exceeded the amoxicillin concentration of 50 µg/kg.

Table 15. LC-MS/MS parameters used for amoxicillin quantifying. (Anonymous, 2015)

Analyte	Ionization mode	Molecular weight	Precursor ion (m/z)	Product ion, m/z (CE, eV)
Amoxicillin	Positive	365.4	366	113(19), 133(31), 349(5)

Table 16. Recoveries and coefficients of variation for amoxicillin in flatfish and eel. (Anonymous, 2015)

Analyte	LOQ (µg/kg)	MRL (µg/kg)	Flatfish		Eel	
			Recovery (%)	CV (%)	Recovery (%)	CV (%)
Amoxicillin	5	25	61	14	63	14
		50	67	9	60	9
		100	115	3	104	10

Three comparative tables summarizing the residue depletion information described above are included in Table 17, and Table 18.

Table 17. Residue depletion studies of amoxicillin in fish treated amoxicillin sodium by i.m. Mean residue concentrations in muscle after end of treatment

Ref.	Fish	Water temp. (°C)	Dose i.m. (mg/kg bw/d)	N° samples/ slaughter day	Mean residue concentrations in muscle after end of treatment (µg/kg)								
					Day 1	Day 2	Day 3	Day 5	Day 7	Day 12	Day 14	Day 21	
Park <i>et.al.</i> , 2013	flatfish	22 ± 3 °C	40	10	--	23 ± 14	--	<0.005 ¹	<0.005	--	<0.005	<0.005	
			single										
		400	10	--	548± 530		57.9 ²	<0.005 ³	--	<0.005	<0.005		
		single											
		40											
	21-22 °C	single (clinical trial)	7	--	--	--	<0.005	--	--	--	--		
	Lim <i>et.al.</i> , (2016)	olive flounder	22 °C ± 1 °C	40	10	--	--	71.2 ± 54.9	55.1 ± 18.1	11.3 ± 6.4	N/D	--	--
				single									
			400	10	--	--	1975.9 ± 639	--	1213.4 ± 199.7	14.2 ± 3.5	--	ND	
			single										
Park <i>et.al.</i> , 2016		23 ± 1 °C	37.5 (7 days)	10	17.1 ^ε	4700 ^ε	--	--	300 ^ε	--	<40 µg/kg (LOQ)	--	

-- data not reported in the manuscript, ¹ all samples <0.005 µg/kg except for one sample with 0.21 µg/kg, ² excluding two samples <0.005 µg/kg, ³ all samples <0.005 µg/kg except for one sample with 0.024 µg/kg, [†] Data from muscle plus skin samples; ND, no detected level.

Table 18. Residue depletion studies of amoxicillin trihydrate in fish orally treated. Mean residue concentrations in muscle after end of treatment

[illegible]

Ref.	Fish	Water temp. (°C)	Dose Oral (mg/kg bw/d)	N° samples/ slaughter r day	Mean residue concentrations in muscle after end of treatment (µg/kg)															
					2 h	6 h	12 h	Day 1	Day 2	Day 3	Day 4	Day 5	Day 7	Day 9	Day 10	Day 12	Day 13	Day 20	Day 21	
Ang <i>et.al.</i> , 2000																				
	Catfish	27.5 + 1.4 °C	110 (1 d)	5	--	--	--	5.4	2.9	<1.2 (LOQ)	--	--	--	--	--	--	--	--	--	--
Chung <i>et.al.</i> , 2006*																				
	Sea bream			5	--	--	--	172 + 53	53 + 29	17 + 6	<10 (LOD)	--	--	--	--	--	--	--	--	--
	rock fish	25 °C		5	--	--	--	131 + 43	38 + 17	10 + 9	<10 (LOD)	--	--	--	--	--	--	--	--	--
	Olive flounder		400 (7 d)	5	--	--	--	137 + 39	42 + 25	12 + 8	<10 (LOD)	--	--	--	--	--	--	--	--	--
	Sea bream			5	--	--	--	96.2 + 2.3	53.8 + 1.4	13.6 + 0.5	7.6 + 0.3	<10 (LOD)	--	--	--	--	--	--	--	--
	Rock fish	25 + 1 °C		5	--	--	--	84.1 + 2	33.9 + 1	10 + 0.4	7.3 + 0.3	<10 (LOD)	--	--	--	--	--	--	--	--
	Olive flounder			5	--	--	--	84.3 + 2.1	55.2 + 1.1	12.6 + 0.5	7.4 + 0.4	<10 (LOD)	--	--	--	--	--	--	--	--
Park <i>et.al.</i> , 2010																				

*No specification of amoxicillin salt is included in the paper

Methods of analysis for residues in tissues

A number of published articles describe methods to determine residues of amoxicillin in fish products: olive flounder, (Chung *et.al.*, 2006, Son, *et.al.*, 2011, Park *et.al.*, 2016, Lim *et.al.*, 2016), rockfish (Chung *et.al.*, 2006), red sea bream (Chung *et.al.*, 2006, Della Rocca, *et.al.*, 2004), grouper (Wang *et.al.*, 2009), bass (Kuo *et.al.*, 2009), pompano (Wang *et.al.*, 2015a, Wang *et.al.*, 2015b), catfish (Ang *et.al.*, 1996, Ang *et.al.*, 1998), tilapia (Luo and Ang, 2000), salmon (Inglis *et.al.*, 1992, Ang *et.al.*, 1996), rainbow trout (Sorensen *et.al.*, 1999),

Screening methods

A microbiological method using *Bacillus stearothermophilus* ATCC 10149 bacteria is described by Della Rocca *et.al.*, 2004, testing amoxicillin residual levels in sea bream. The LOD for that test was reported to be 2 µg/L.

Charm 7000 is described by Inglis *et.al.*, 1992, as an assay based on a competitive binding reaction between the antibiotic tested and a tracing agent radio-labelled member of the same antibiotic family tested. This test cannot differentiate between members of the same antibiotic family. Salmon tissue samples spiked with amoxicillin (between 80 and 5 µg/L) were used to prepare a standard curve. ¹⁴C- counts per minute (cpm) were obtained when measuring the different amoxicillin concentrations found in spiked salmon samples. The percentage reduction in cpm is obtained as the difference between the zero-antibiotic ¹⁴C-count and the sample ¹⁴C-count as a percentage of the zero count. If the level of drug increased then, the percent of ¹⁴C-cpm decreased, with a maximum of detection of 0.08 and a minimum of 0.005 ppm. Low counts obtained in the results, indicate that little labelled antibiotic is found because most of receptor sites are blocked by a high level of antibiotic in the sample. Instead, high counts indicate low antibiotic in the sample thus, most sites are available to react with the labelled substance.

Della Rocca, *et.al.*, 2004 also describe the Charm II test. Although the sensitivity of this test was determined as 5 µg/L, in the context of the analytical methods employed, the serum concentrations were found to be underestimated by this test. Additionally, this test is not specific to differentiate between members of the same antibiotic family and it is not specifically developed to measure actual concentrations but it can be considered as screening test.

Quantitative methods

Single-residue methods

In a study, Chung *et.al.*, (2006) performed the amoxicillin residue analysis on fish muscle tissues with the HPLC system. The description of the clean-up procedure of amoxicillin for fish muscle is described. Standard curve was created with concentrations from 50 to 100 µg/L. Results indicated good linearity with r^2 value of 0.993. The recovery rates ranges of amoxicillin in the samples were 84.3-101.3 % and 75.0-91.5 % for the concentration of 50 µg/kg and 100 µg/kg, respectively. Table 19 shows the recoveries of amoxicillin from fortified muscle tissue samples. The results indicate recovery ranges according fish species of 80.9 – 100.2 % (olive flounder), 75.0- 88.2 % (rockfish) and 78.4 – 101.3 % (red sea bream).

Table 19. Muscle tissues sample recoveries from olive flounder (*Paralichthys olivaceus*), Rockfish (*Seebastes schlegeli*) and red sea bream (*Pagrus major*) (Chung *et.al.*, 2006)

Fish species	N° of samples	Fortified concentration (µg/g)	Recovery (%)	
			Range	Mean
<i>P. olivaceus</i>	3	0.05	85.3-100.2	97.3
		0.1	80.8-91.5	86.7
<i>S. schlegeli</i>	3	0.05	88.2-99.6	95.7
		0.1	75.0-85.9	82.9
<i>P. major</i>	3	0.05	92.1 – 101.3	96.5
		0.1	78.4-89.2	87.8

In a different study for olive flounder, Son, *et.al.*, (2011), describe the use of HPLC to determine amoxicillin concentrations in muscle. A standard calibration curve (dilution points of 50, 100, 200, 500, and 1000 µg/kg, for the HPLC analysis) was created with an amoxicillin regression coefficient (r^2) at 0.9966. Table 20 shows the recovery values, LOD and LOQ obtained.

Table 20. Amoxicillin recovery values, LODs and LOQs obtained in olive flounder muscle samples. (Son *et.al.*, 2011)

Antibiotic	Fortified concentration (µg/g)	Recovery (%)	SD	CV	LOD (µg/kg)	LOQ (µg/kg)
Amoxicillin	0.5	103.8	7.59	7.32	2.1	7
	1.0	10.8	9.14	9.08		

SD : Standard deviation, CV : Coefficient of variation, LOD : Limit of detection, LOQ: Limit of Quantification

Park *et.al.*, 2016 describe the use of a validated HPLC method also for the determination of amoxicillin residues in tissues of olive flounder. The HPLC method validation for analysis of amoxicillin in tissue samples was performed using tissue samples spiked with amoxicillin sodium at various concentrations ranges. Accuracy and precision data of amoxicillin from combined muscle plus skin tissues samples are listed in Table 21. HPLC method for analysis of amoxicillin in combined muscle plus skin tissues samples was specific and sensitive.

Table 21. HPLC validation data for olive flounder muscle plus skin samples spiked with amoxicillin sodium at different concentrations (Park. *et.al.*, 2016).

Analyte	Spiked concentration (mg/g)	Precision (CV)	Accuracy (%)
Amoxicillin	2	0.09	92.25
	1	3.26	93.79
	0.1	0.39	96.04
	0.05	3.7	93.31
	0.02	8.4	95.40

CV : coefficient of variation

The use of an HPLC method with solid phase extraction and UV detection was described by Wang *et.al.*, 2009, to determine the amoxicillin concentrations in muscle of grouper. A recovery range of 81 to 98 % was obtained from grouper muscle spiked with 1, 5 and 10 µg/g (Table 22). Accuracy, precision and linearity were determined intra-day and inter-day (Table 23).

Table 22. Amoxicillin recovery values obtained in grouper muscle samples (Wang *et.al.*, 2009)

Fortified concentration (µg/g)	Recovery (%) Mean± SD
1.0	89.95 ± 7.71
5.0	97.67 ± 8.39
10.0	81.13 ± 7.82

Table 23. Amoxicillin inter and intra-day precision data in grouper muscle samples (Wang *et.al.*, 2009)

Fortified Concentration (µg/g)	n	Inter-day		Intra-day	
		Measured (µg/kg) Mean ± SD	CV (%)	Measured (µg/kg) Mean ± SD	CV (%)
1.0	5	0.93 ± 0.11	11.3	0.92 ± 0.09	10.2
5.0	5	4.42 ± 0.53	11.9	4.68 ± 0.48	10.3
10.0	5	8.12 ± 0.88	10.8	8.35 ± 0.67	8.1

Similarly, Kuo *et.al.*, 2009 described the use of a solid-phase extraction and high performance liquid chromatography (HPLC) method with UV detector to determine the amoxicillin levels in bass muscle. For working standard solutions, the validated concentration ranges of this method (where amoxicillin calibration curve showed linearity) were 0.01 µg/ml to 10 µg/ml, instead, for muscle they were 40 to 100 µg/kg (linear correlation coefficient of 0.9970). Ten concentration points were employed to construct the calibration curves by plotting the peak area versus the theoretical concentrations. The method of least squares linear regression analysis was utilized to determine the slope, intercept, and the correlation coefficient. The amoxicillin concentration of the quality control samples and unknown samples were calculated using the calibration curves. Amoxicillin concentrations of 1, 5, and 10 µg/g were added to bass muscle to investigate the recoveries. A recovery range of 74 to 83 % of the targeted value with coefficients of variation of 10 % or less, was obtained. Mean absolute recoveries were 78 %. Accuracy, precision and linearity were determined intra-day and inter-day. The LOD was calculated as 40 µg/kg and the LOQ was 100 µg/kg.

Wang *et.al.*, 2015a, used a reverse phase HPLC to investigate the amoxicillin residues concentrations in serum and tissues samples from pompano. They describe the sample preparation procedure of muscle extraction and clean-up of fish tissue for amoxicillin detection. Samples were prepared for HPLC analysis using solid phase extraction steps. For the assay

validation, a calibration curve with 10 concentration points was obtained plotting the peak area versus the theoretical concentrations. Intra-assay (within day) and inter-assay (between days) variations were analyzed. The standard curve showed linearity from 40 to 10000 µg/kg (linear correlation coefficient of 0.9991). LOD was calculated to be 40 and LOQ 100 µg/kg. A recovery range of 74.3 to 83.4 % was obtained when muscle tissue samples were spiked with 50, 100, 500, and 1000 µg/kg of amoxicillin. The coefficients of variation were less than 10 %. The recovery average was 73.5 ± 3.7 %. Replicate measurements of three known pompano samples with amoxicillin concentrations of 1000, 5000, and 10000 µg/kg were measured to evaluate the precision of the assay. The mean \pm SD and CV (%) were obtained by replicate analyses in the same run (intra-day) and in separate runs (inter-day). The CV values were considered under an acceptable level of precision (below 11.6 %).

Wang *et.al.*, 2015b describe a solid phase extraction and HPLC with UV detection method to determine amoxicillin residues from liver and kidney tissues samples in pompano. As described above, Kuo *et.al.*, 2009, use similar approach in bass muscle tissue. The LOD and LOQ were calculated 40 and 100 µg/kg, respectively. Replicate measurements ($n = 6$) of three spiked pompano liver samples at concentrations of 100, 500 and 1000 µg/kg were used to determine the precision of the assay. Intra-assay (within day) and inter-assay (between days) variations were analyzed. The relative standard deviation (RSD) for all the samples was lower than 12.1 %. The absolute recovery was calculated of 73 – 84 % (mean 78 ± 4 %).

A study was conducted to develop a highly sensitive analytical method using reversed-phase liquid chromatographic (LC) with fluorescence detection to determine amoxicillin concentrations in catfish and salmon tissues, at ≤ 10 ppb (Ang *et.al.*, 1996). Recoveries of spiked amoxicillin from salmon muscle tissues were generally lower than the values obtained for catfish because of interfering compounds. This method is applicable for determining amoxicillin residues at 5 µg/kg in both, salmon and catfish muscle tissue. Average recoveries of > 80 % for catfish and > 75 % for salmon muscle tissues, with coefficients of variation (CV) of < 6 %, were obtained after amoxicillin was spiked at 2.5 to 20 µg/kg, respectively (Table 24 A and B). The limit of detection (LOD) was 0.5 µg/kg for catfish and 0.8 µg/kg for salmon muscle tissue. The limits of quantitation (LOQ) were 1.2 and 2 µg/kg for catfish and salmon, respectively.

Table 24. Amoxicillin recoveries from spiked muscle tissues samples from (A) catfish and (B) salmon (Ang. *et.al.*, 1996)**A****B**

Amount spiked ng/g	Within-day recovery, %				Amount spiked ng/g	Within-day recovery, %			
	<i>n</i>	Mean	SD	CV ^a		<i>n</i>	Mean	SD	CV ^a
20	7	78.0	2.29	2.93	20	7	76.2	3.21	4.21
10	7	81.2	2.64	3.25	10	7	79.5	2.40	3.02
5.0	7	81.4	2.52	3.46	5.0	7	77.7	2.79	3.59
2.5	7	81.9	2.91	3.55	2.5	5	67.0	3.65	5.45
Control	7	ND ^b	--	--	Control	7	ND ^b	--	--
Mean		80.02	2.48	3.24	Mean		75.1	3.01	4.07

Amount spiked ng/g	Day-to-day recovery, %				Amount spiked ng/g	Day-to-day recovery, %			
	<i>n</i>	Mean	SD	CV ^a		<i>n</i>	Mean	SD	CV ^a
20	7	82.0	2.91	3.55	20	5	80.2	4.50	5.61
10	7	81.4	2.80	3.43	10	5	79.2	2.43	3.07
5.0	7	80.6	3.04	3.77	5.0	5	81.2	3.58	4.41
Control	7	ND ^b	--	--	Control	5	ND ^b	--	--
Mean	7	81.4	2.92	3.58	Mean		79.9	3.50	4.36

^aCV= (SD/mean) x 100^bND= None detected (<0.5 ppb)

In a different study, also in catfish (*Ictalurus punctatus*), the correlation between a liquid chromatographic (LC) method and a microbial inhibition (MI) method for the analysis of amoxicillin residues muscle samples was investigated by a bridging study (Ang *et.al.*, 1998). The intra-laboratory validation with fortified samples showed that the LC method has satisfactory accuracy and precision for determining amoxicillin residues in catfish and salmon muscle samples at 10 µg/kg. Both methods were compared for determination of fortified and incurred samples. Muscle samples were fortified with amoxicillin to concentrations of 5, 10, 20, 40, 50 and 100 µg/kg. Two laboratories analyzed the samples concurrently. Amoxicillin recoveries from the fortified fish muscle samples analyzed by MI method showed mean recoveries of > 90 % with coefficients of variations (CV) of < 7 % and no difference between analytical days or the two analysts performing the study. The LOD for the MI assay was 4 µg/kg and the LOQ ranged from 11.6 to 13.0 µg/kg. The MI method recovery range (96.2 – 102 %, with CVs ranging from 2.9 to 4.87 %) seems to be higher than those of LC analysis (78.0 – 82.0 %). One of the reasons for the differences might be due to preparing solutions for calibrations curves. Intra-laboratory method validation was also done for MI. MI showed the lowest mean recovery (82.8 %) and the highest CV (11.7 %), however, these values are still within the FDA acceptability criteria for analytical methods for residues analysis. After statistical analysis, no significant differences were found between the methods. Although, for amoxicillin residues at < 10 µg/kg, LC method was shown to be more specific and with better

sensitivity than MI, the values obtained by the two methods can be considered equivalent for residue levels of 20 to 100 µg/kg. LC was able to determine residues at 5 µg/kg which was below the detection limit of the MI method. Analysis of the samples by LC did not show interferences.

A study was conducted by Luo and Ang, (2000) to improve the determination of amoxicillin residues in muscle tissues by solid-phase extraction (SPE) and liquid chromatography with fluorescence detection, requiring less flammable solvent use. Muscle tissues from various animal species, one of them is tilapia, were extracted with phosphate buffer followed by the modified SPE procedure for cleanup and concentration prior to LC-fluorescence analysis. Frozen tissue samples were thaw and fortified to yield final concentrations of 5, 10 or 20 µg/kg at room temperature (23 °C) for 30 to 60 minutes. This method is a modification of the method described by Ang *et.al.*, 1996. The main difference is in the SPE procedure involving the use of 50 % aqueous acetonitrile solution instead of 100 % acetonitrile, thus a cleaner sample eluate is obtained and can be used directly in the derivatization step without the extraction step. The tilapia amoxicillin recovery data is listed in Table 25. Considering the three fortification levels, the mean recoveries for tilapia ranged from 92.5 to 95.4 %. The subsamples had a variability (expressed as RSDs) ranging from 1.26 to 3.56 %. Studies also were conducted to evaluate potential interfere with other animal drugs, such as other β-lactam, erythromycin, lincomycin and streptomycin. No interference peaks were observed. This method is specific for amoxicillin. In this study, the modified procedure was tested for fortified tissues. Additionally, as described above, Ang *et.al.*, 2000, demonstrated that the LC method with fluorescence detection was satisfactory for the determination of incurred amoxicillin residues within the range tested. Thus, an LC-MS/MS confirmatory test was developed and confirmation of amoxicillin residues was demonstrated in incurred fish samples containing 50 and 297µg/kg of amoxicillin.

Table 25. Comparison of amoxicillin recoveries from fortified animal muscle tissues (Luo and Ang, 2000)

Fortification, µg/kg (ppb)	Amoxicillin recovery, % Tilapia		
	Mean	SD	RSD
0	ND	—	—
5	95.4	1.2	1.26
10	92.5	1.6	1.73
20	92.6	1.4	1.51
Average	93.5	1.4	1.50

1 Standard deviation (n = 5).

2 Relative standard deviation, % (SD/mean × 100 %).

3 Nondetectable (<0.8 µg/kg).

A LC method based on solid-phase extraction was developed for the determination of amoxicillin residues in muscle tissues of rainbow trout (*Oncorhynchus mykiss*), avoiding liquid-liquid extraction with ozone-depleting agents or highly flammable solvents (Sorensen *et.al.*, 1999). Thirty rainbow trout (100 g) were acclimatized in basins with water temperature

of 11.8 ± 0.5 °C, pH 7.5 ± 0.14 and oxygen content > 8.5 mg/L. Fish were dosed with 100 mg amoxicillin/kg bw/day by automatic machines for 5 days. Trout samples were taken before and after the administration period and stored at -20 °C. Additionally, muscle samples were spiked with amoxicillin to levels of 10, 30, 100 and 200 µg/kg to determine the repeatability, intra-laboratory reproducibility and recovery of the method. The LOD was determined to be 2.9 µg/kg and the LOQ 4.1 µg/kg. The mean recovery in muscle was independent of concentration level and was calculated to be 80.5 % (Table 26). Calibration curves were linear with coefficient of determination exceeding 0.9994. Shortly after amoxicillin administration, amoxicillin residues were detected in muscle tissue, but not 3 weeks later (n=10) or before treatment (n=2). Muscle tissue samples (n=10) at 18 to 22 h after administration dose showed a concentration range of 180-9020 µg/kg (average 1770 µg/kg). For incurred residues, the relative repeatability standard deviations were observed as 6.1 and 6.7 % at the measured concentrations of 11 and 143 µg/kg, respectively.

Table 26. Relative repeatability standard deviation (RSD_r), relative intra-laboratory reproducibility standard deviation ($RSDR_{intra}$), and amoxicillin recovery from spiked muscle tissue samples from rainbow trout^a (Sorensen *et.al.*, 1999)

Spiked µg/kg	level,	RSD_r , %	$RSDR_{intra}$, %	Recovery, %	
				Mean	SD ^b
10		4.35	5.45	80.3	3.6
30		2.85	5.80	82.0	4.5
100		3.57	4.27	79.2	2.7
200		4.05	5.94	80.6	2.6

^a Amoxicillin concentration was determined in duplicate at each spiked level. One double determination was performed at each level on each of the 8 different days. Blank sample material used for spiking was different each day.

^b Standard deviation of 8 mean results obtained by double determinations.

Amoxicillin concentrations in olive flounder muscle were determined using a validated LC-MS/MS method (Lim *et.al.*, 2016). Although the Korean Food Standards Codex states that the use of acetonitrile as solvent is optimum for the resolution of the amoxicillin peak by this method, the authors found that it was best resolved using methanol as the solvent. Additionally, the run time was set to 15 minutes to minimize interference between the target peak and the sample matrix. The calibration curve was linear in the range of 5 - 100 ng/ml ($r^2 = 0.9997$); The LOD was 2.36 ng/ml and the LOQ was 7.16 ng/ml. Amoxicillin average recoveries from muscle, determined by LC-MS, after doses of 10 and 100 ng/ml were 98.0 and 111.0 %, respectively.

Multi-residue methods

A number of multi-residues analytical methods have been reported for amoxicillin (Freitas *et.al.*, 2014, Kaufmann *et.al.*, 2011, Fernandez-Torres *et.al.*, 2011a, Fernandez-Torres *et.al.*, 2011b, Fernandez-Torres *et.al.*, 2010, Juan-Garcia *et.al.*, 2007, Smith *et.al.*, 2009, Maggi *et.al.*, 2012, Yipel *et.al.*, 2016). Also, the main amoxicillin metabolite, amoxicilloic acid, can be simultaneously determined by High Performance Liquid Chromatography (HPLC)

(Fernandez-Torres *et.al.*, 2010; Fernandez-Torres *et.al.*, 2011a) and HPLC with Diode Array-Fluorescence (HPLC-DAD-FLD) (Fernandez-Torres *et.al.*, 2011b).

Table 27 includes a summary of amoxicillin multi-residue methods found during the search in the literature available in the public domain

Table 27. Summary of amoxicillin multi-residue methods

Ref	Method	Drugs	Matrices	Amoxicillin	
				LOD/LOQ	Recovery (%)
Freitas <i>et.al.</i> , 2014	Ultra-high performance liquid chromatography with tandem mass spectrometry (UPLC-MS/MS)	41 antibiotics including amoxicillin	Muscle Gilthead sea bream	CC β = 64.2 μ g/kg CC α = 78.5 μ g/kg	105
Kaufmann <i>et.al.</i> , 2011	Improved high resolution mass spectrometry	More than 100 different veterinary drugs including amoxicillin	Pork muscle meat, bovine kidney, bovine liver, fish and honey	No reported for fish	No reported for fish
Fernández-Torres <i>et.al.</i> , 2011 ^a	Enzymatic-microwave assisted extraction and high-performance liquid chromatography–mass spectrometry	11 antibiotics including amoxicillin	Hake, anchovy, mussel and wedge sole	Anchovy LOD = 16 μ g/kg LOQ = 55 μ g/kg CC α = 9 μ g/kg CC β = 15 μ g/kg	Anchovy 50 μ g/kg = 86 \pm 3 100 μ g/kg = 82 \pm 4 200 μ g/kg =89 \pm 4
				Wedge sole LOD = 16 μ g/kg LOQ = 53 μ g/kg CC α = 9 μ g/kg CC β = 14 μ g/kg	Wedge sole 50 μ g/kg = 92 \pm 4 100 μ g/kg = 92 \pm 6 200 μ g/kg =93 \pm 4
				Hake LOD = 16 μ g/kg LOQ = 57 μ g/kg CC α = 9 μ g/kg CC β = 15 μ g/kg	Hake 50 μ g/kg = 84 \pm 5 100 μ g/kg = 84 \pm 9 200 μ g/kg =89 \pm 5
Fernandez-Torres <i>et.al.</i> , 2010	Enzymatic probe sonication extraction prior to high-performance liquid chromatography			Anchovy = NQ Wedge sole LOD = 130 μ g/kg LOQ = 440 μ g/kg CC α = 120 μ g/kg CC β = 180 μ g/kg	Anchovy = NQ Wedge sole 50 μ g/kg = 58.7 \pm 4.9 100 μ g/kg = 61.1 \pm 1.3 200 μ g/kg = 58.1 \pm 4.3
				Hake LOD = 110 μ g/kg LOQ = 390 μ g/kg CC α = 110 μ g/kg CC β = 160 μ g/kg	Hake 50 μ g/kg = 67.9 \pm 4.7 100 μ g/kg = 65.9 \pm 3.9 200 μ g/kg = 68.3 \pm 2.5

Fernández-Torres <i>et al.</i> , 2011b	Reversed-phase High-Performance Liquid Chromatography with Diode Array-Fluorescence (HPLC-DAD-FLD) Detection			Anchovy = NQ Wedge sole LOD = 110 µg/kg LOQ = 340 µg/kg CCα = 130 µg/kg CCβ = 180 µg/kg Hake LOD = 160 µg/kg LOQ = 510 µg/kg CCα = 170 µg/kg CCβ = 290 µg/kg	Anchovy = NQ Wedge sole 50 µg/kg = 70.0±3.5 100 µg/kg = 77.7±2.2 200 µg/kg = 78.5±3.5 Hake 50 µg/kg = 52.6±7.2 100 µg/kg = 50.3±1.6 200 µg/kg = 50.7±5.0
Juan-García <i>et al.</i> , 2007	Capillary electrophoresis-mass spectrometry CE-IT-MS/MS	12 antibacterial drugs including amoxicillin	Fish (<i>Sparus aurata</i>)	LOD = 10 µg/kg LOQ = 30 µg/kg	50 µg/kg = 76±17 100 µg/kg = 79±12 200 µg/kg = 77±8
Smith <i>et al.</i> , 2009	Liquid Chromatography-ion trap mass spectrometry	38 compounds including amoxicillin	Trout, salmon, catfish, and tilapia	Intermediate confirmation limits 0.1ppm	Recoveries from fortified samples estimated 10 % at 0.1ppm
Maggi <i>et al.</i> , 2012	On-line Solid-Phase Extraction Coupled to Liquid Chromatography-Ion Trap Tandem Mass Spectrometry	8 penicillins including amoxicillin	catfish	CCα = 0.45 µg/kg CCβ = 1.8 µg/kg	71.7 (at 2 µg/kg)
Yip <i>et al.</i> , 2016	LC-MS/MS	37 antibiotics including amoxicillin	Rainbow trout, sea bass and gilthead sea bream	LOD = 0.216 µg/kg LOQ = 0.72 µg/kg CCα = 63.4 µg/kg CCβ = 72.9 µg/kg	61.36

LOD = limit of detection, LOQ = limit of quantitation, CCα = decision limit, CCβ = detection capability, NQ = Non-quantifiable

Stability of residues during storage

During stability studies plasma samples stored at -80 °C showed no significant difference until 2 months (Seo *et al.*, 2014).

In a preliminary storage stability study, catfish muscle samples containing 2 concentrations of incurred amoxicillin residues, 8.75 and 41.5 µg/kg, were analyzed by LC revealing that after 2 months storage at -70 °C, no significant changes (<5 %) were found. Another study from the same group tested fortified muscle samples for storage stability, analyzing the samples by LC after 1 and 7 days at -70 °C. Data showed that no changes due to storage were found. Some decrease in amoxicillin concentrations stored for 4 to 7 days was found by MI method,

however, the difference was < than 10 % of day 1, which could be due to day to day variation (Ang and Luo, 1998).

In another study to investigate the stability during sample storage, Sorensen *et.al.*, (1999) determined that amoxicillin concentrations in trout tissues stored at $-20^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 10 months declined 11 % (a mean content of 199 $\mu\text{g/kg}$) from the original level, indicating degradation of the compound. Fish samples spiked to a level of 80 $\mu\text{g/kg}$ and stored at $-20^{\circ}\text{C} \pm 2^{\circ}\text{C}$ showed higher losses (See figure 5 on Sorensen *et.al.*, 1999). Final sample extract of muscle spiked to amoxicillin concentration of 50 $\mu\text{g/kg}$ were stable for at least 12 days if stored at $10^{\circ}\text{C} \pm 2^{\circ}\text{C}$.

Appraisal

Amoxicillin is an old compound with a long history of use and has been previously reviewed by the Committee.

In fish, amoxicillin hydrate is administered by oral route at dose of 20 - 40 mg/kg bw fish, once or twice a day, for a period of 4 to 7 days in yellowtail against pasteurellosis. The optimum dose is 40 mg/kg bw and the higher dose is 80 mg/kg bw of fish. Based on the dose regimen approved in a Member State for yellowtail, amoxicillin is administered to flounder, rockfish and red sea bream. Amoxicillin sodium is also administered by intramuscular route as a dose of 12.5 or 40 mg/kg bw of fish, once a day, in olive flounder, against streptococcosis. An association of amoxicillin (tri)hydrate in combination with florfenicol is administered as upper intramuscular injection at a single dose of 10 mg/kg in olive flounder (*Paralichthys olivaceus*), against Streptococcus and Edwardsiella.

No data was found about the metabolism of amoxicillin in fish.

Eleven non-GLP studies investigating the pharmacokinetic data on amoxicillin are available for a variety of fish species using various routes of administration and experimental formulations: eel (Jeon *et.al.*, 2010), flatfish (Park, *et.al.*, 2013), sea bream (Della Rocca *et.al.*, 2004), pompano (Wang *et.al.*, 2015a), salmon (Brown and Grant, 1992; Inglis *et.al.*, 1992; Inglis *et.al.*, 1993), and olive flounder (Seo *et.al.*, 2014; Park *et.al.*, 2016; Lim *et.al.*, 2016). Three of these studies were conducted with amoxicillin sodium after intramuscular administration and the others with amoxicillin trihydrate.

Bioavailability of amoxicillin in fish after oral administration was found to be low. Rate of absorption is dependent on formulation and route of administration. The data analyzed suggest that the use of a different formulation (amoxicillin trihydrate vs. amoxicillin sodium) accounts in part for the differences in pharmacokinetic parameters. Amoxicillin sodium was much rapidly absorbed than amoxicillin trihydrate, following a single intramuscular dose of either, 12.5, 40, 80 or 125 mg/kg bw. Amoxicillin concentrations in plasma were higher at higher doses by i.m. administration if amoxicillin sodium was used in the treatment instead of amoxicillin trihydrate.

No amoxicillin radiolabelled residue depletion data were available for evaluation.

Residue depletion studies with non-radiolabeled amoxicillin are available for different fish species: flatfish (Park *et.al.*, 2013), grouper (Wang *et.al.*, 2009), bass (Kuo *et.al.*, 2009), catfish (Ang *et.al.*, 2000), salmon (Inglis *et.al.*, 1992), pompano (Wang *et.al.*, 2015b), sea bream (Della Rocca, *et.al.*, 2004), rockfish and sea bream (Chung *et.al.*, 2006; Park *et.al.*, 2010); and olive flounder, (Chung *et.al.*, 2006; Park *et.al.*, 2010; Son *et.al.*, 2011; Park *et.al.*, 2016; Lim *et.al.* 2016). These studies were performed after oral administration of amoxicillin trihydrate or intramuscular administration of amoxicillin sodium.

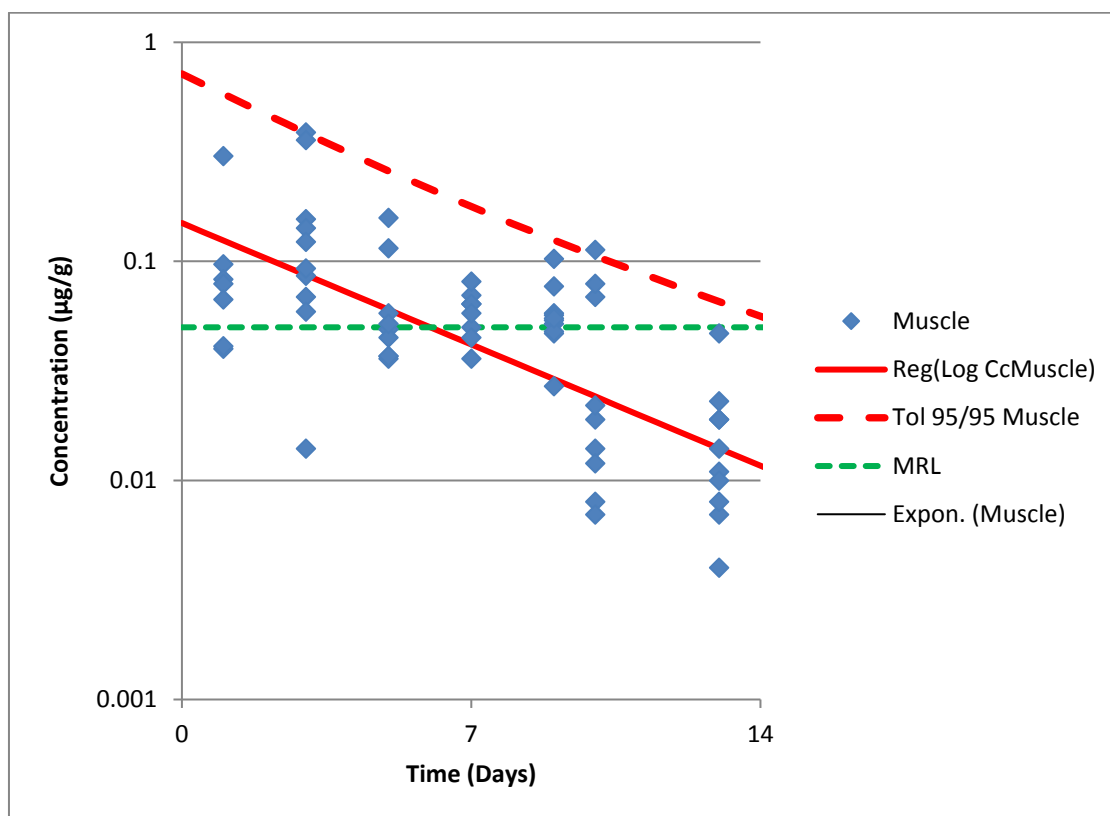
In all studies, amoxicillin residues deplete rapidly. Residues in muscle are low, and persist few days after the last administration. For many studies, the sampling time frames are too long to permit a detailed analysis of residue depletion in muscle. The data obtained from peer reviewed papers were mainly available as means and standard deviations or graphically. Additional information, including individual animal data, from only one study was submitted for independent analysis by the committee. Studies performed in olive flounder published in peer review paper, allow estimating withdrawal times.

No information on the position of sampling sites in relation to injection sites was provided in the residue depletion studies. However, considering the fish anatomy, the high solubility of amoxicillin sodium and the rapid absorption rate, the Committee considered it is likely that concentrations at injection sites are similar to those in other muscle tissues.

Qualitative and quantitative single or multi-residue methods are available to determine residues of amoxicillin, the main microbiologically active residue identified in muscle and muscle + skin. Validated analytical methods, HPLC/UV, HPLC/Fluorescence and LC-MS/MS were reviewed. Multi-residue LC-MS/MS methods for the screening, quantification and confirmation of amoxicillin residue in muscle of different animal species including fish were reported. These methods are appropriate for the monitoring of amoxicillin residues in fish muscle and fillet.

Using the data from the study of Son (2011) after 7 oral doses of amoxicillin (40 mg/kg) in olive flounder (*Paralichthys olivaceus*), the upper one-sided 95 % confidence limits over the 95th percentile of residue concentrations was calculated for muscle (Figure 15). The committee estimated the median concentration (10 µg/kg) at 15 days when the upper one-sided 95 % confidence limit is close to the previously established MRL of 50 µg/kg for muscle of cattle, sheep and pig.

Figure 15. Residue depletion curves of amoxicillin in olive flounder based on data from Son *et.al.* 2011



Dietary Exposure

Chronic dietary exposure assessment

Dietary exposure to amoxicillin is considered to occur only through its use as a veterinary drug. There is no registered use for amoxicillin as a pesticide.

At its 75th meeting the Committee considered the use of amoxicillin in cattle, sheep and pigs. The Committee did not calculate an estimated daily intake (EDI) for amoxicillin at that time, owing to the small number of quantified residue data points. Using the model diet of 300 g muscle, 100 g liver, 50 g kidney, 50 g fat and 1.5 litre of milk with the MRLs recommended at the 75th JECFA meeting, the theoretical maximum daily intake (TMDI) is 31 µg/person per day, which represents 74 % of the upper bound of the ADI.

Based on the hazard profile of the compound, estimates of acute dietary exposure are required for the general population and children. Chronic dietary exposure estimates for children and shorter than lifetime dietary exposure are not required. Dietary exposure was estimated based on the potential occurrence of amoxicillin residues in a single food (finfish muscle and skin in natural proportion) after 7 days of treatment and 15 days withdrawal time (217 degree-days). Consumption inputs were based on the highest reported values available within the fish category (i.e., marine fish consumptions for means, freshwater fish consumption for the 97.5th percentile). While Codex MRLs have been confirmed for edible tissues of cattle, sheep and

pigs, and milk of cattle and sheep, median residue concentrations for these potential sources of exposure and the MRLs were based on the LOQ. While additional dietary exposure from these sources cannot be excluded, it is not possible to quantify it at this time.

Based on a median residue concentration of 10 µg/kg at a withdrawal time of 15 days, the GECDE for the general population is 0.14 µg/kg bw per day, which represents 7 % of the upper bound of the microbiological ADI of 0.002 mg/kg bw (Table 28).

In addition to the accepted GECDE methodology, further calculations were carried out. Instead of using the highest mean and the highest 97.5th percentile consumption across surveys, the calculations were carried out using the mean and the highest reliable percentile for each individual national survey from available data sets (CIFOCCOss). The mean of 25 estimates was 0.024 µg/kg bw per day (1 % of the upper bound of the ADI), with a range of 0.0006-0.14 (0.03-7 % of the upper bound of the ADI). All of the individual national estimates are summarized in Table 29.

These results suggest that the GECDE is sufficiently conservative, as it exceeds or is equal to all national estimates of chronic dietary exposure.

Acute dietary exposure assessment

No assessment of acute dietary exposure has previously been carried out for amoxicillin.

Based on a 95/95 UTL residue concentration of 50 µg/kg at a withdrawal period of 15 days, the GEADE for the general population is 1.4 µg/kg bw, which represents 28 % of the microbiological ARfD of 0.005 mg/kg bw. The GEADE for children is 1.6 µg/kg bw, which represents 31 % of the microbiological ARfD (Table 30).

Table 28. The global estimated chronic dietary exposure (GECDE) to amoxicillin median residues (15 days withdrawal) in the general population

Category	Type	Median residue concentration ¹ (µg/kg)	Mean consumption ² , whole population, g/kg bw per day	HRP consumption ³ , consumers only, g/kg bw per day (percentile)	MR:TR ratio	Exposure µg/kg bw per day mean	HRP	GECDE ⁴ µg/kg bw per day	% ADI
General Population									
Fish and seafood	Fish	10	0.39	13.6 (97.5)	1	0.004	0.14	0.14	
TOTAL								0.14	7

¹Median concentration 15 days post treatment ²highest mean consumption figures based on whole population considered from the available dataset ³highest reliable percentile (HRP) food consumption figures based on consumers only considered from the available dataset. If >180 consumers were represented the percentile is 97.5, if 61-180 consumer are represented the percentile is 95, if 31-60 consumers are represented the percentile is 90. If less than 30 consumers are represented only the mean consumption value is used ⁴GECDE is the sum of the highest exposure at the highest reliable percentile of consumption for a food and the mean dietary exposures of the other foods

Table 29. National estimates of chronic dietary exposure to amoxicillin median residues (15 days withdrawal) in adults or the general population, based on GECDE methodology.

Country	Population group	Study	Estimate of chronic dietary exposure (µg/kg bw per day)	% ADI	Main contributing food
Bangladesh	Adult women	Harvest_2007/8	0.018	0.9	Freshwater fish
Belgium	Adults	Diet_National_2004	0.021	1.0	Cod
Brazil	General population	Brazilian Institute of Geography and Statistics	0.138	6.9	Freshwater fish
Burkina Faso	Adult women	Harvest_2010	0.001	0.0	Freshwater fish
China	General population	2002 China Nutrition and Health Survey	0.048	2.4	Freshwater fish
Czech Republic	Adults	SISP04	0.015	0.8	Herring
Denmark	Adults	Danish_Dietary_Survey	0.007	0.4	Plaice
Finland	Adults	FINDIET_2007	0.026	1.3	Pacific salmon
France	Adults	INCA2	0.011	0.5	Cod
Germany	Adults	National_Nutrition_Survey_II	0.025	1.3	Herring
Hungary	Adults	National_Repr_Surv	0.019	0.9	Carps
Ireland	Adults	NSIFCS	0.013	0.6	Pacific salmon
Italy	Adults	INRAN_SCAI_2005_06	0.020	1.0	Cod

Country	Population group	Study	Estimate of chronic dietary exposure (µg/kg bw per day)	% ADI	Main contributing food
Japan	General population	DSFFQ_FI	0.011	0.5	Marine fish
Latvia	Adults	EFSa_TEST	0.012	0.6	Herring
Netherlands	Adults	DNFCS_2003	0.007	0.3	Pacific salmon
South Korea	General population	KNHNES	0.053	2.6	Pollack
Spain	Adults	AESAN	0.026	1.3	Marine fish
Spain	Adults	AESAN_FIAB	0.029	1.4	Marine fish
Sweden	Adults	Riksmaten_1997_98	0.005	0.2	Herring
Thailand	General population	FCDT	0.009	0.4	Freshwater fish
Uganda	Adult women	Harvest_2007	0.015	0.7	Freshwater fish
United Kingdom	Adults	NDNS	0.013	0.6	Pacific salmon
USA	Childbearing women	What We Eat in America - Food Commodity Intake Database	0.028	1.4	Freshwater fish
USA	General Population	What We Eat in America - Food Commodity Intake Database	0.028	1.4	Freshwater fish
Mean			0.024	1.2	
Minimum			0.001	0.0	
Maximum			0.138	6.9	

Table 30. The global estimate of acute dietary exposure (GEADE) to amoxicillin, based on 95/95 UTL residues (15 days withdrawal) in the general population and in children

Category	Type	Highest concentration ¹ (µg/kg)	residue (µg/kg)	97.5 th food consumption ² , consumers only (1 day), g/kg bw per day	GEADE, µg/kg bw per day	% ARfD
General population						
Fish and seafood	Fish	50		28	1.4	28
Children						
Fish and seafood	Fish	50		31	1.6	31

¹Highest concentration 15 days post treatment ²highest 97.5th food consumption figures based on consumers only (1 day)

Maximum Residue Limits

In order to address the ambiguity of the terms ‘flatfish’ and ‘finfish’ the Committee decided that for the purpose of this report the term finfish would include all fish species. In recommending MRLs for amoxicillin in finfish, the Committee considered the following factors:

An ADI of 0-0.002 mg/kg bw was established by the Committee based on a microbiological endpoint.

The less than lifetime exposure scenarios will be covered by the acute exposure assessment.

- An ARfD was established at 0.005 mg/kg bw based on a microbiological endpoint,
- Amoxicillin is authorized for use in at least two fish species in one Member State. The withdrawal periods of approved use are unknown.
- It is also used for other fish species in other Member States
- Data on metabolism of amoxicillin in fish were not provided.
- Amoxicillin is not widely distributed in fish tissues, not lipophilic and does not accumulate in fat,
- Pharmacokinetics and residue depletion curves were similar for different fresh and salt water representative fish species belonging to different orders (Salmoniformes, Perciformes, Siluriformes, Anguilliformes and Pleuronectiformes).
- Amoxicillin is the only microbiologically active residue and is suitable as a marker residue.
- Suitable validated routine analytical methods were available for monitoring purposes and can be applied to different fish species.
- The Codex MRL of 50 µg/kg established for cattle, sheep and pig muscle is suitable for the control of residue in muscle and fillet of finfish species.
- The Committee recommended MRLs of 50 µg/kg for amoxicillin in muscle, or muscle and skin in natural proportions of fin fish.

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Ampicillin

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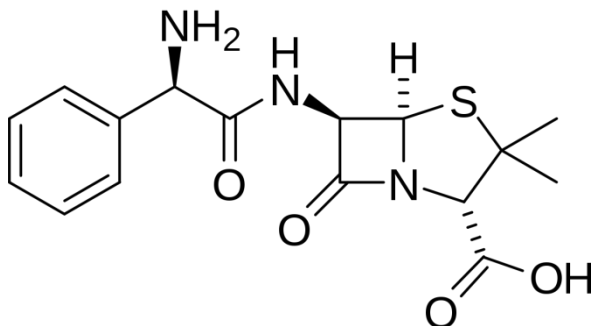
International Non-proprietary Names (INN): Ampicillin

Synonyms: Adobacillin, Albipen, Ampicillin A, Aminobenzylpenicillin, Ampicillin acid, Totacillin

IUPAC Name: (2S,5R,6R)-6-([(2R)-2-amino-2-phenylacetyl]amino)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid

Chemical Abstract Service No.: Ampicillin 69-53-4, Ampicillin sodium 69-52-3. Ampicillin trihydrate 7177-48-2

Structural formula:



Molecular formula: Ampicillin $C_{16}H_{19}N_3O_4S$, Ampicillin sodium $C_{16}H_{18}N_3NaO_4S$, Ampicillin trihydrate $C_{16}H_{19}N_3O_4S \cdot 3H_2O$

Molecular weight: Ampicillin 349.41 g/mol, Ampicillin sodium 371.387 g/mol, Ampicillin trihydrate 403.45 g/mol

Other information on identity and properties

Pure active ingredient: Ampicillin

Appearance: white or almost white crystalline powder

Melting point: Ampicillin 199-202 °C

Solubility: Ampicillin was reported to be sparingly soluble in water at room temperature (Merck Index, 2006), and practically insoluble in acetone and in ethanol 96 %. It is soluble in diluted acidic solution or hydroxide alkaline solutions.

Ampicillin sodium : Easily soluble in water, quite soluble in acetone

Ampicillin trihydrate : poorly soluble in water, practically insoluble in ethanol 96 %.

Ampicillin trihydrate is soluble in diluted acidic solution or hydroxide alkaline solutions

pH: Ampicillin 3.5 to 5.5, Ampicillin sodium 8.0 to 10.0, Ampicillin trihydrate 3.5 to 5.5

Optical rotation: Ampicillin monohydrate, $[\alpha]_D^{21} = + 281^\circ$ ($c = 1$ in H_2O), Ampicillin sodium $[\alpha]_D^{20} + 283.1^\circ$ ($c = 0.2$ in H_2O), Ampicillin anhydrous $[\alpha]_D^{20} + 287.9^\circ$ ($c = 1$ in H_2O)

Stability: Ampicillin trihydrate was reported as stable at 25 °C at 43 % and 81 % relative humidity for 6 weeks, with little change in either moisture content or activity.

Dissociation constant: $pK_a = 2.5; 7.3$ (23 °C)

Background

Ampicillin is authorized for use as a veterinary drug against bacterial infections in a wide range of companion and food animals, including fish species used for food production (unspecified yellowtail, olive flounder). The clinical indications for ampicillin cover a variety of infections, including those of the respiratory and urinary tracts, septicemia and enteric infections.

Ampicillin is produced by the acylation of 6-aminopenicillanic acid with D-(-)- α -phenylglycine by either microbiological or chemical synthesis. Ampicillin is bactericidal and has a similar mode of action to that of benzylpenicillin. Although it has a broader spectrum of activity, covering several additional gram-positive and gram-negative organisms. Ampicillin may have a synergistic action with aminoglycosides and with the β -lactamase inhibitors clavulanic acid and sulbactam (Foulds, 1986; Barnhart, 1989).

Ampicillin is classified by WHO as critically important antimicrobial in human medicine (CIA) and, therefore, prudent use in animal husbandry is recommended (WHO, 2011). In addition, OIE classified Ampicillin as critically important antimicrobial in veterinary medicine (VCIA) (OIE, 2015)

Ampicillin has not previously been evaluated by the Committee. In 2016 the sponsor requested ADI and MRL establishment in flat fish muscle and skin in natural proportions. At the 23rd session of the Codex Committee on Residues of Veterinary Drugs in Foods (CCRVDF) the request was extended to MRLs for fin fish muscle and skin in natural proportions accordingly.

Data on ampicillin in flat fish were submitted to JECFA. These included information on the two ampicillin formulations, including a list of trade names and approved uses in fish, monitoring data on occurrence of ampicillin in aquaculture products (Anonymus, 2015), as well as studies on pharmacokinetics (Jung *et.al.*, 2012; Park, *et.al.*, 2013) and residue depletion of ampicillin in flat fish (Son *et.al.*, 2011; Toxicological Research Center, 2014; Park, *et.al.*,

2013). English translations for the original studies were provided. In addition, raw data were provided by the sponsor on request for one residue and one pharmacokinetic study. Information on established withdrawal periods for authorized products is missing. Proposals were made by the authors of the studies regarding possible withdrawal periods.

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

- Ampicillin AND fish AND residue
- Ampicillin AND fish AND kinetics
- Ampicillin AND fish AND withdrawal
- Ampicillin AND fish AND metabolism
- Ampicillin AND fish AND analytical method
- Ampicillin AND (salmon OR trout) AND (residue OR kinetics OR withdrawal OR metabolism OR analytical method)

The following criteria were applied to filter the articles with regard to the assessment to be conducted:

Inclusion criteria	Exclusion criteria
Any article focusing on	Any article focusing on
- ampicillin concentrations in plasma of fish	- bacteria resistance to ampicillin
- ampicillin concentrations in edible tissues of fish	- ampicillin use in food animal species other than fish
- residue determination in fish plasma / tissue	- kinetics/residues of antimicrobials other than ampicillin (and do not include ampicillin for comparison)
- bioavailability of ampicillin residues in fish	- environmental issues
No restrictions concerning year of publication	Any article dealing with ornamental fish

The literature search resulted in 36 potentially relevant articles. A thorough review of the articles led to further exclusion of 20 articles based on the above-mentioned criteria. Sixteen articles were considered relevant and were used in the monograph.

None of the studies used for the evaluation indicated they were performed under GLP conditions. For one residue study from the published literature, only the abstract and tables were available in English. As only three more residue studies were available, it was decided to include these data in the evaluation.

An MRL for ampicillin of 50 µg/kg for edible tissues was established in some member states for all food producing species. In another member state, MRLs were established for edible

tissues from salmoniformes (50 µg/kg), anguilliformes (50 µg/kg), perciformes (60 µg/kg) and other fish (50 µg/kg) (The Japan Food Chemical Research Foundation, 2006). In an additional member state, an MRL of 50 µg/kg was established for fish tissues (Anonymous, 2011).

Residues in food and their evaluation

Conditions of use

Ampicillin is a penicillin β -lactam antimicrobial agent used in the treatment of bacterial infections caused by susceptible, usually gram-positive, organisms. Ampicillin has *in vitro* activity against gram-positive and gram-negative aerobic and anaerobic bacteria. The bactericidal activity of ampicillin results from the inhibition of cell wall synthesis and is mediated through ampicillin binding to penicillin binding proteins (PBPs). Ampicillin is stable against hydrolysis by a variety of β -lactamases, including penicillinases, and cephalosporinases and extended spectrum β -lactamases.

In aquaculture, ampicillin is used against microorganism causing pseudotuberculosis, vibriosis, streptococciosis, and edwardsiellosis (Son *et.al.*, 2011). In at least one member state veterinary medicines containing ampicillin hydrate are used for treatment of pasteurellosis in yellowtail (not further unspecified) and ampicillin sodium is used for treatment of streptococcosis of olive flounder (*Paralichthys olivaceus*).

Dosage

In one member state, ampicillin hydrate (CAS No. 7177-48-2) is licensed as an oral powder for use in fish at a dosage of 5 to 20 mg per kg bodyweight (bw) once or twice a day for five days. Ampicillin sodium (CAS No. 69-52-3) is licensed for treatment via upper intramuscular injection at a single dose of 20 mg per kg bw.

Pharmacokinetics and metabolism

Pharmacokinetics in laboratory animals

The pharmacokinetic parameters for ampicillin in laboratory animals are summarized in Table 1.

Absorption, distribution and excretion

Mice

In a non-GLP reported study, plasma concentrations of ampicillin trihydrate were measured in Tg-rasH2 mice treated orally with 3000 mg/kg bw for 26 weeks (Adachi *et.al.*, 2002). No information on the analytical method used is provided. Blood was sampled 1 and 3 h after the first (day 1) and the final dose (after 26 weeks). Repeated dosage during 26 weeks resulted in a 2 to 3 times higher plasma concentration 1 hour after dosing, in males 24.53 µg/mL (\pm 0.33) and 58.06 µg/mL (\pm 12.07), and in females 23.53 µg/mL (\pm 1.89) and 79.57µg/mL (\pm 14.85) after first and final dose, respectively. The values 3h after dosing were measured in males to

2.64 µg/mL (\pm 1.81) and 8.56 µg/mL (\pm 6.29), and in females to 8.56 µg/mL (\pm 6.29) and 8.48 µg/mL (\pm 9.54) after first and final dose, respectively. The three-hour values appear to be incorrectly reported as they are the same for females after the first dosing and for males after the final dosing.

A biphasic elimination was found when mice were treated i.v. with ampicillin trihydrate at a dose of 10 mg/kg bw (English *et.al.*, 1976). Analysis was conducted using a microbiological assay with *Micrococcus luteus*, ATCC 9341. The half-life for the first alpha distribution phase was 1.18 min and for the second beta phase was 23.20 min AUC was $296.82 \pm 23.44 \mu\text{g} \times \text{h/mL}$. In another non-GLP reported study, the half-life for ampicillin was 1.12 h and AUC was $4.39 \pm 0.40 \mu\text{g} \times \text{h/mL}$ in mice (males and females) treated orally with sultamicillin (20 mg/kg bw), a prodrug to ampicillin (English *et.al.*, 1984).

Rats

Distribution of ampicillin sodium was studied in rats (n=10/group) treated orally via stomach tube with 100 mg/kg bw during 24 h, in a non-GLP reported study (Acred *et.al.*, 1962). Ampicillin was assayed by a cup-plate technique using *Sarcina lutea* as test organism. Except for stomach and intestine the highest amounts were found in liver and kidney, 15.82 and 13.22 µg/g, respectively. Ampicillin was found in the other tissues in amounts of 4.42 µg/g in serum, 2.22 µg/g in spleen and 6.17 µg/g in lungs. The highest concentration (µg/g wet weight) was measured after 0.5 h and then declined in liver, spleen, kidneys, lungs, stomach, small intestine, carcass and serum, but increased during 2 and 4 h in caecum, colon and in faeces. In urine the concentration increased successively until 12 h (up to 6.63 %) and then decreased. Less than 0.09 µg/g were found after 24 h in all tissues except in small intestine, colon and faeces. Four h after administration the recovery was 43 to 65 % indicating that ampicillin is excreted rather quickly in rats. In conclusion, ampicillin is evenly distributed in all tissues, but with highest concentrations in kidney and liver.

Rats (males) were treated orally with 10 or 12 mg/kg bw ampicillin trihydrate in a non-GLP reported study (English *et.al.*, 1984). An agar diffusion bioassay made use of *Micrococcus luteus* ATCC 9341 for analysis of ampicillin. The oral absorption was 23.0 % with a C_{max} of $0.86 \pm 0.21 \mu\text{g/mL}$ and AUC of $1.80 \pm 0.01 \mu\text{g} \times \text{h/mL}$. The highest concentrations in tissues of ampicillin were found in liver and kidney followed by plasma, lung, spleen and muscle. In rats (males) treated orally with sultamicillin, a prodrug to ampicillin which is hydrolysed to ampicillin and sulbactam, the half-life for ampicillin was 0.75 h, AUC was calculated to $2.76 \pm 0.11 \mu\text{g} \times \text{h/mL}$ and oral absorption was 56.7 %. It was shown that ampicillin given as a prodrug had a 2.5 times increased oral absorption.

Rabbits

Rabbits (5/group) were treated orally with 100 mg/kg bw of ampicillin (Acred *et.al.*, 1962). The highest amount was measured after 1 h in blood, 2.16 µg/mL, and successively decreased to 0.06 µg/mL of blood after 4 h.

Dogs

Dogs (5 dogs/group) were treated orally with 20 mg/kg bw and blood samples were collected every 30 min up to 5.5 h after administration and also after 24 h (Acred *et.al.*, 1962). The highest concentration in serum was found to be 7.23 µg/mL after 1 h, which declined to 0.11 µg/mL after 5.5 h and to 0 µg/mL after 24 h.

In dogs (beagle dogs, males) treated orally with 20 mg/kg bw of sultamicillin, a prodrug to ampicillin, the half-life for ampicillin was 0.98 h and AUC was $19.32 \pm 2.06 \mu\text{g} \times \text{h/mL}$ (English *et.al.*, 1984).

Table 1 . Pharmacokinetic parameters for ampicillin in laboratory animals

Species	Route of administration	Absorption %	Time h	Dose mg/kg bw	t _{1/2} h	C _{max} µg/mL	AUC (0-∞h) µg x h/mL	Reference
Mice	oral		4	20	1.12		4.39±0.40	English <i>et.al.</i> , 1984
Mice	i.v.			10	α: 0.02 β: 0.39		296.8±23.4	English <i>et.al.</i> , 1976
Rats	oral	23	24	10-12	0.75	0.86±0.21	1.80±0.01	English <i>et.al.</i> , 1984
Dogs	oral		4	20	0.98		19.32±2.06	English <i>et.al.</i> , 1984

Humans

In a non-GLP reported study 10 resp. 7 humans were treated with a capsule with 250, 500, 750 or 1000 mg ampicillin trihydrate and serum concentrations were measured 0.5, 1, 2, 4 or 6 h after dosing (Knudsen *et.al.*, 1961). Ampicillin concentrations were determined by the cup-plate biological assay method using *Sarcina lutea* ATCC 9341 as test organism. The results for C_{max}, t_{max} and excretion in urine are listed in Table 2. The serum concentrations declined to 0.17, 0.2, 0.3 and 0.63 µg/mL after 6 h. This study shows that ampicillin is rapidly absorbed after oral administration, with a maximum concentration in serum after 1 to 2 h. 6 h after dosing, approximately 30 % of the given dose, was excreted in urine and the concentrations in serum was low.

Table 2 . Pharmacokinetic parameters for ampicillin sodium or ampicillin when administered to humans

Formulation	Route	Excretion in urine (%)	Time (h)	Dose (mg/person)	t _{max} (h)	C _{max} µg/mL	AUC (0-24 h) µg h/mL	References
-Trihydrate	Oral fasting	37.1±12.1	8	500	1.9	5.9±2.1	19.8 ± 5.5	Eshelman and Spyker, 1978
-Trihydrate	Oral nonfasting	26.8 ±8.3	8	500	2.4	4.6±1.6	13.7 ± 4.9	
-H	Oral	25.1		500				Haginaka and Wakai, 1987
Metabolite II	-	3.29						
Metabolite III	-	1.82						
Metabolite IV	-	0.37						
-Trihydrate	Oral	33	6	250	1	2.19		Knudsen <i>et.al.</i> , 1961
-Trihydrate	Oral	25	6	500	2	3.8		
-Trihydrate	Oral	28	6	750	2	5.1		
-Trihydrate	Oral	33	6	1000	2	6.79		

Metabolite II: (5R,6R)-ampicilloic acid

Metabolite III: (5S,6R)-ampicilloic acid

Metabolite IV: ampicillin piperazine-2,5-dione

The effect of food on pharmacokinetic parameters of ampicillin in humans (16 males) was investigated in a double blind crossover study (Eshelman and Spyker, 1978). Samples were assayed by the large plate microbiological method, with *Staphylococcus aureus* (ATCC 6538P) as the assay organism resp. by the small plate method, with *Sarcina lutea* (ATCC 9341) as the assay organism. Fasting compared to non-fasting before oral administration of 500 mg ampicillin capsules resulted in a faster absorption rate (0.34 h ± 0.36 vs 1.43 h ± 0.61) as well as higher C_{max} (5.9 µg/mL ± 2.1 vs 4.6 µg/mL ± 1.6) and shorter time to C_{max} (1.86 h ± 0.3 vs 2.40 h ± 0.41), higher urine recovery (37.1 % ± 12.1 vs 26.8 % ± 8.3) and a higher AUC (19.8 ± 5.5 h × µg/mL vs 13.7 h × µg/mL ± 4.9).

One human was treated with 500 mg ampicillin and urine was sampled up to 8 h after administration (Haginaka and Wakai, 1987). HPLC was used for the determination of ampicillin and its metabolites ((5R,6R)-ampicilloic acid, (5S,6R)-ampicilloic acid, ampicillin piperazine-2,5-dione). Fraction of urinary excretion of ampicillin and metabolites were measured to 25.1 % for ampicillin, 3.29 % for metabolite II, 1.82 % for metabolite III and 0.37 % for metabolite IV. The mean residence time from administration to excretion in urine was approximately 2 to 3.5 h for parent compound and metabolites (Table 2).

Ampicillin is known to cross the placenta and also to be excreted in breast milk during feeding (Nathanson *et.al.*, 2000).

Pharmacokinetics in Food-producing Animals

Olive flounder (*Paralichthys olivaceus*)

In a study in olive flounder (*Paralichthys olivaceus*, mean bw 300 g), ampicillin was administered via three routes, i.e. by oral, intramuscular and water bath treatment (Jung *et.al.*, 2012). There were 9 treatment groups of 25 fish each. Fish were managed in a flow-throw water tank at a temperature of 20 ± 1 °C.

The first three groups were treated with 10, 20, and 40 mg ampicillin per kg bw via medicated feed (single treatment), one pellet each inserted into the mouth of unanaesthetized fish. For water bath treatment, another three groups were placed for one hour into three different water tanks, which were prepared with ampicillin doses of 10, 20, and 40 mg/kg bw. The last three groups were treated once via intramuscular injection into the muscle near the dorsal fin with 5, 10, or 20 mg/kg bw.

Blood samples were taken as samples at 13 time points between 1 h and 360 h after dosing from the caudal vein. Concentrations of residues in plasma were determined by HPLC-UV (LOD 50 µg/kg, no LOQ given).

Ampicillin concentrations in blood after oral administration, intramuscular injection and water bath treatment are shown in Figure 1, Figure 2, and Figure 3 respectively. For oral administration of ampicillin, all groups reached the maximum blood concentration at 10 h after dosing, reaching 3.62 ± 0.97 µg/mL for 10 mg/kg, 5.20 ± 0.70 µg/mL for 20 mg/kg, and 11.18 ± 0.87 µg/mL for 40 mg/kg. Ampicillin was eliminated more rapidly at lower doses, and could not be detected in the blood at 144 h, 360 h, and 360 h, respectively. For intramuscular injections of ampicillin, all groups reached the maximum concentration at 5 h after administration, reaching 6.92 ± 1.29 µg/mL for 5 mg/kg bw, 9.89 ± 2.22 µg/mL for 10 mg/kg bw, and 19.85 ± 2.97 µg/mL for 20 mg/kg bw. Ampicillin could not be detected in the blood at 216 h, 264 h, and 264 h, respectively. With water bath treatment, all groups reached the maximum concentration at 3 h after administration, reaching 4.39 ± 1.10 µg/mL for 10 mg/kg bw, 9.57 ± 1.51 µg/mL for 20 mg/kg bw, and 11.61 ± 1.95 µg/mL for 40 mg/kg bw. Ampicillin could not be detected in the blood at 264 h, 264 h, and 360 h, respectively.

Figure 1. Mean plasma concentration (N=5) of ampicillin in olive flounder after single oral feeding with dose of 10, 20 and 40 mg/kg body weight at 20 ± 1.0 °C. (Jung *et.al.*, 2012)

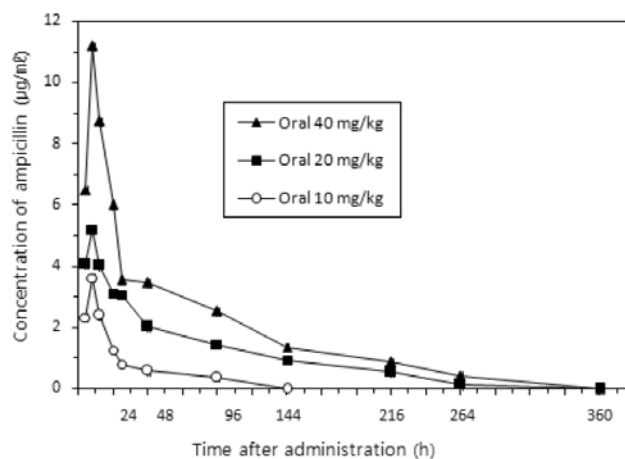


Figure 2. Mean plasma concentration (N=5) of ampicillin in olive flounder after single intramuscular injection with dose of 5, 10 and 20 mg/kg body weight at 20 ± 1.0 °C. (Jung *et.al.*, 2012)

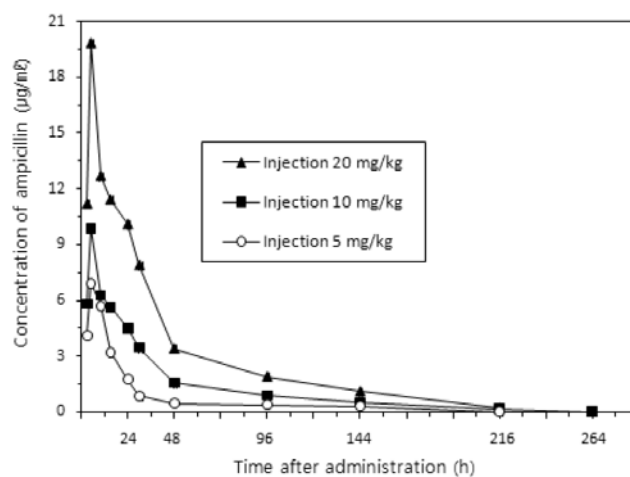
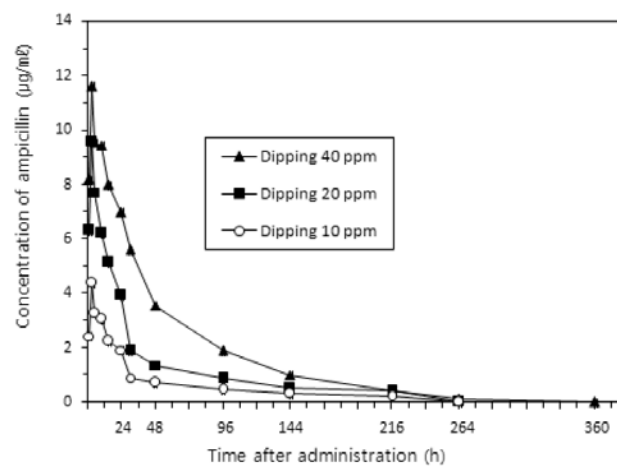


Figure 3. Mean plasma concentration (N=5) of ampicillin in olive flounder after single dipping with dose of 10, 20 and 40 ppm for 1 h at 20 ± 1.0 °C. (Jung *et.al.*, 2012)



Flatfish

In a study in flatfish (species not specified, mean bw 150 g), conducted at a water temperature of 22 ± 3 °C, fish were treated with a single intramuscular injection at dosages of 5, 10, 20 or 40 mg/kg bw (Park *et.al.*, 2013). Ampicillin concentrations in plasma were measured by HPLC/UV (no information on LOQ available) and pharmacokinetic parameters were derived based on a non-compartmental model. Pharmacokinetic parameters are summarized in Table 3. Plasma concentrations in flatfish after intramuscular administration of a number of doses of amoxicillin are shown in Figure 4.

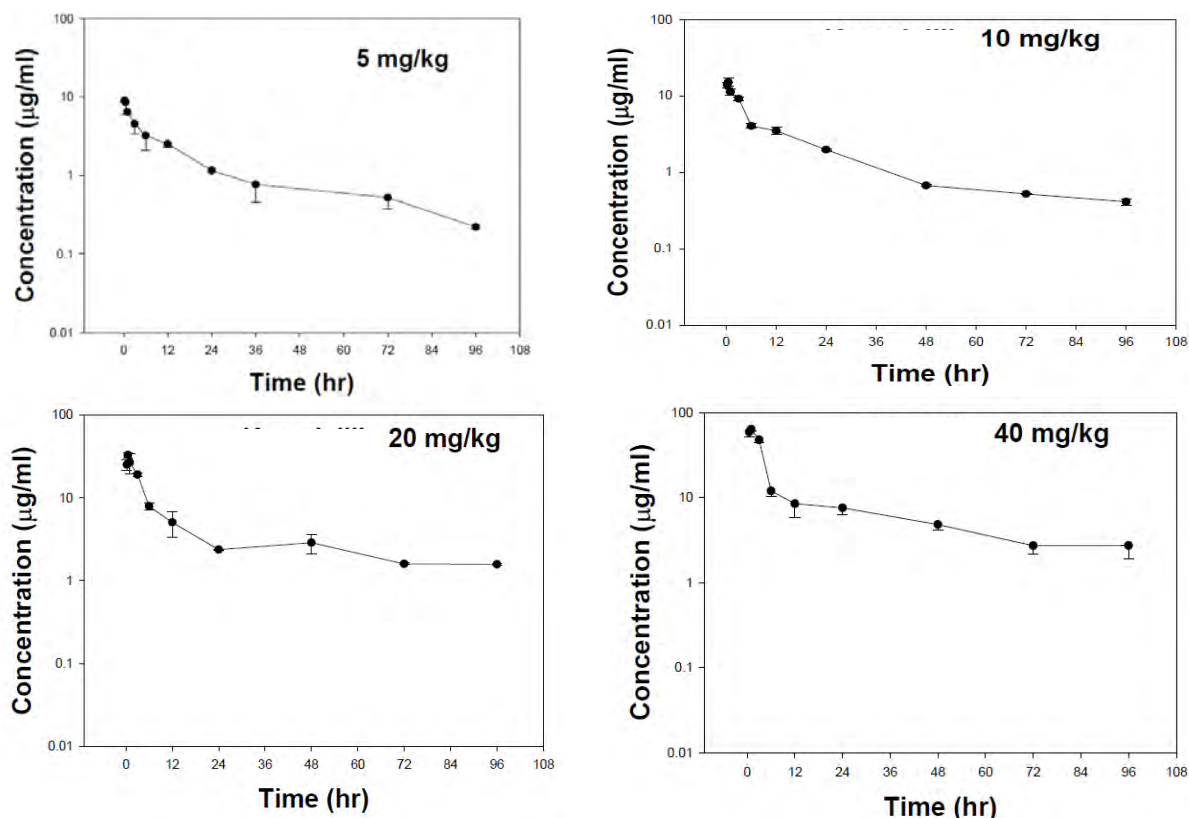
Table 3. Pharmacokinetic parameters of ampicillin in blood from flatfish treated at different doses (non-compartment model) (Park *et.al.*, 2013)

Pharmacokinetic parameter	Doses			
	5 mg/kg bw	10 mg/kg bw	20 mg/kg bw	40 mg/kg bw
Number of animals by analysis point	5	5	5	5
Maximum concentration C_{max} (µg/mL)	9.1	15.1	32.9	63.4
Time to reach the maximum concentration t_{max} (h)	0.25	0.50	0.50	1.00
Elimination half-life $T_{1/2}$ (h)	33.4	67.8	44.0	41.5
Area Under Curve (to the measurement point) AUC_{0-t} (µg/mL*h)	113.1	164.5	349.5	708.2
Area Under Curve (to infinity) $AUC_{0-\infty}$ (µg/mL*h)	123.9	24.5	449.4	871.8
Average Residence Time (to infinity) $MRT_{0-\infty}$ (h)	35.0	55.4	57.4	50.3

In the dosage group treated at 5 mg/kg bw, blood concentration reached the maximum concentration (9.05 ± 0.47 µg/mL) in 15 min and decreased to 3.25 ± 1.14 µg/mL after 6 h. After a single administration of ampicillin at the dose of 10 mg/kg bw, concentration in plasma reached the maximum (15.06 ± 1.86 µg/mL) in 30 min and decreased to 4.03 ± 0.26 mg/mL after 6 h. The plasma concentration reached the highest concentration (32.86 ± 2.35 µg/mL) in 30 min after administration of ampicillin at a dosage of 20 mg/kg bw, but remained at 5.06 ± 1.74 µg/mL until after 12 h. After that, the concentration was maintained at 1.5 µg/mL or more until the final analysis time of 96 h. The plasma concentration reached the highest concentration (63.38 ± 3.40 µg/mL) in one hour after administering 40 mg/kg bw of ampicillin, and remained at approximately 5 µg/mL until after 48 h. After that, the concentration was maintained at 2.73 ± 0.82 µg/mL or more until the final analysis time of 96 h.

Elimination half-lives were 33.4 h, 67.8 h, 44.0 h and 41.5 h after treatment at doses of 5, 10, 20 or 40 mg/kg bw (5 samples per dosage group), respectively. The time to reach maximum concentrations increased with dose from 0.25 h to 1.00 h.

Figure 4. Plasma concentrations in flatfish after intramuscular administration of 5, 10, 20 resp. 40 mg amoxicillin per kg bw (Park *et.al.*, 2013)



Predictive approaches using structure activity relationships or in silico tools to predict ADME properties

Not available.

Metabolism in Laboratory Animals

Rats

In a non-GLP reported study intended to evaluate a new analytical method, wistar rats (N=3, males) were treated i.v. with ampicillin sodium (1) at a dose of 100 mg/kg bw (Haginaka *et.al.*, 1987). Blood samples were taken after 0, 20, 40, 60, 120 and 240 min and plasma samples were prepared. Bile and urine samples were sampled directly from the bile duct or ureter followed by HPLC analyses with pre and post column derivatization. The following parameters were calculated for ampicillin in rat plasma; Area Under Curve (AUC) 5.89 ± 2.68 mg min/mL, Mean residence time (MRT) 59.3 ± 5.7 min, volume steady state (Vss) 1130 ± 415 mL/kg, Clearance (CLt) 19.4 ± 8.2 mL/min/kg. The metabolites (5R,6R)-ampicilloic acid (2) and its epimer (5S,6R)-ampicilloic acid (3) and ampicillin piperazine-2,5-dione (4) were found in plasma, bile and urine. Plasma levels in rats for the metabolites was <0.5 µg/mL. The excretion in bile and urine was 75.6 % of administered dose of which 69.5 % was unchanged ampicillin and 6.1 % was from metabolites. In general, only limited details were reported.

Metabolism following oral dosing in laboratory rats was not found in the literature.

Metabolism in Humans

The metabolism of ampicillin was studied in two non-GLP reported studies. Ampicillin is metabolized to ampicillin piperazine-2,5-dione, (5R,6R)-ampicilloic acid and its epimer (5S,6R)-ampicilloic acid. A method for analysing the metabolites in human urine has been developed (Haginaka *et.al.*, 1986).

One human was treated with 500 mg ampicillin and urine was sampled up to 8 h after administration (Haginaka & Wakai 1987). The fraction of the analysed metabolites in urine was 3.29 % of (5R,6R)-ampicilloic acid (2) and 1.82 % of its epimer (5S,6R)-ampicilloic acid (3), 0.37 % of ampicillin piperazine-2,5-dione (4) and 25.1 % ampicillin after 8 h (Table 2).

Metabolism in Food Producing Animals

No studies on metabolism in fish species are available, i.e. it is unknown whether the metabolites found in mammals would also be present in fish treated with ampicillin.

As degradation of ampicillin into ampicilloic acid (5S,6R ampicilloic acid and 5R,6R ampicilloic acid) takes place in acid aqueous medium (de Nascimento *et.al.*, 2013), it cannot be excluded that this would also occur in fish tissues. Ampicilloic acids do not have microbiological activity but are of relevance for allergic reactions and are therefore of toxicological relevance. No information concerning the ratio of ampicillin to ampicilloic acids in muscle from treated fish is available.

Comparative metabolism

Published literature reports, non GLP reported, have shown that the metabolites found in rats are also found in humans after oral administration of ampicillin. Apart from the parent compound the metabolites (5R,6R)-ampicilloic acid (II) and its epimer (5S,6R)-ampicilloic acid (III), and ampicillin piperazine-2,5-dione (IV) were found. Only 30 % was identified/characterized in human urine but in rats approximately 75 % has been characterized. No radiolabelled studies have been found in any species. As only 30 % was characterized in urine from humans it is not known if other metabolites are present.

Tissue residue depletion studies

Radiolabelled residue depletion studies

No residue depletion studies using radiolabelled ampicillin in fish were available for evaluation.

Residue depletion studies with unlabeled drug

The residue depletion studies using unlabeled ampicillin were not reported to be GLP compliant. Three studies provided by the sponsor and one additional study from the literature search were included in the assessment. Additionally, the sponsor provided monitoring data for several fish species.

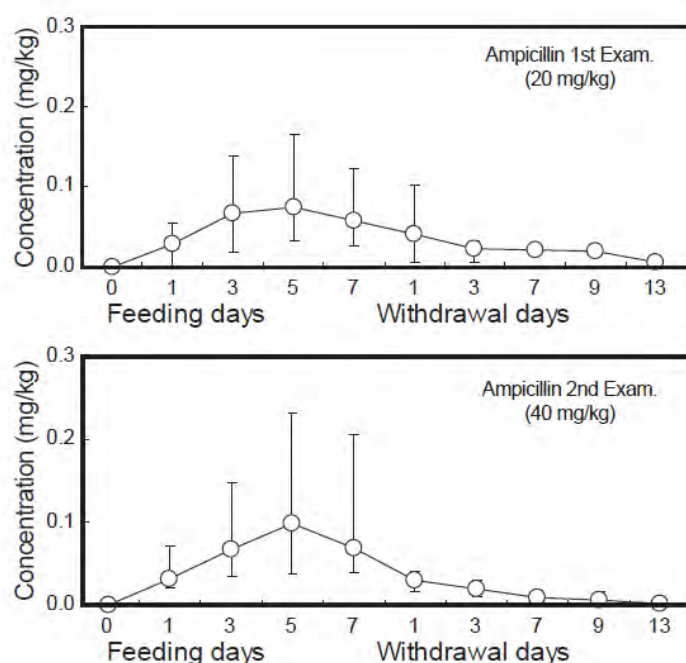
Studies on residue depletion after oral use

The depletion of ampicillin from muscle tissues of olive flounder (*Paralichthys olivaceus*) was studied at two temperatures and two different dosages (Son *et.al.*, 2011). Healthy olive flounders (550 ± 150 g, 34 ± 5 cm) without a history of infection or receiving antibiotics were held in tanks. Fish were treated via medicated feed at a dose of 20 mg/kg bw per day for seven consecutive days at a water temperature range of 13.4-15.6 °C and at a dose of 40 mg per kg bw per day for seven consecutive days at a water temperature range of 16.8-21.4 °C.

Each ten samples were taken at day 0 before treatment, at days 1, 3, 5, and 7 during treatment and at days 1, 3, 7, 9, and 13 post-treatment. An HPLC-UV method was used to analyse the samples (LOQ 5 µg/kg) and validation data were available. At the request of JECFA, the sponsor provided data that were used to calculate summary statistics (Figure 5).

At the lower dose, ampicillin residue concentrations were highest on day 1 after final treatment, at a mean of 41 µg/kg in muscle. On day 3, the mean concentration fell to 23 µg/kg, and by day 13, the ampicillin concentration was below the LOQ. At the higher dose of 40 mg/kg bw, the residual amount of ampicillin was 30 µg/kg on day 1 of withdrawal, by day 3 the concentration was reduced to 19 µg/kg, and by day 9 the concentration of ampicillin was below the LOQ.

Figure 5. Concentration changes of ampicillin in the muscle of olive flounder (*P. olivaceus*) after oral administration of ampicillin (Son *et.al.*, 2011). The upper and under bar indicate the minimum and maximum range of ampicillin concentration in the muscle of olive flounder.



In another study in olive flounder (*Paralichthys olivaceus*), rockfish (*Sebastes schlegeli*), and red sea bream (*Pagrus major*) residue depletion after 5 days treatment with medicated feed at a dose of 100 mg/kg bw/day at water temperatures of 25 °C was investigated (Cho *et.al.*, 2006).

Only the abstract and the tables from this study were available in English. Fish were sampled at days 1, 2, 3, and 4 after treatment (Table 4). For analysis an HPLC method was used (detection method unknown, no LOQ, but recovery values of 94-98 % and 83-88 % for the concentrations of 50 µg/kg and 100 µg/kg, respectively, are reported).

Ampicillin concentrations detected on day 1 after treatment were 143, 138, 187 µg/kg in the muscle of olive flounder, rockfish, and red sea bream, respectively. After a withdrawal period of 3 days, muscle concentrations were 16, 12, and 21 µg/kg in the olive flounder, rockfish, and red sea bream, respectively. Ampicillin was not detectable in any muscle sample 4 days following withdrawal of the medicated feed. From results of the present study, a withdrawal period of 5 days was proposed for 5 days treatment with medicated feed at a dose of 100 mg/kg bw per day

Table 4. Concentration of ampicillin in muscle of fishes after administration of 100 mg/kg bw in feed for 5 days

Tissue	No. of Samples	Residue concentrations after treatment (µg/kg)			
		Day 1	Day 2	Day 3	Day 4
<i>P. olivaceus</i>	5	143 ± 38	52 ± 22	16 ± 6	ND
<i>S. schlegeli</i>	5	138 ± 54	45 ± 19	12 ± 4	ND
<i>P. major</i>	5	187 ± 42	63 ± 28	21 ± 7	ND

ND: Not detected

Studies on residue depletion after intramuscular use

Residue depletion was investigated in flatfish (species not specified, mean bw 150 ± 20 g) held in culturing water at 22 ± 3 °C (Park *et.al.*, 2013). Two groups of fish were treated with 40 mg ampicillin per kg bw or 200 mg ampicillin kg bw via single intramuscular injection. Samples from each dosage group (10 fish) were taken at days 2, 5, 7, 14, 21, and 30 after administration. Location of sampling site in relation to injection site was not specified. Residue concentrations were measured using an LC-MS/MS analysis (no validation data available).

Ampicillin concentrations in flatfish muscle are shown in Table 5. Residue concentrations at both doses continuously decrease with time. Mean residue concentrations were 35.5, 10.1 and 1.1 µg/kg at the dose of 40 mg/kg bw and 422.7, 44.6, and 12.7 µg/kg at the dose of 200 mg/kg bw at days 23, 5, and 7 after treatment, respectively.

Table 5. Ampicillin concentrations in flatfish muscle after single intramuscular treatment with 40 and 200 mg/kg bw (Park *et.al.*, 2013)

Days after administration	Ampicillin concentrations (µg/kg)							
	40 mg/kg				200 mg/kg			
	No.	Conc.	No.	Conc.	No.	Conc.	No.	Conc.
2	1	13.5	6	34.9	1	1,266.7	6	435.2
	2	22.3	7	84.3	2	303.4	7	163.9

	3	17.6	8	22.5	3	318.8	8	521.6
	4	46.3	9	77.2	4	333.7	9	742.4
	5	11.6	10	25.1	5	78.2	10	63.2
	1	8.41	6	5.34	1	18.6	6	123.6
	2	1.10	7	7.30	2	41.7	7	44.2
5	3	2.45	8	22.6	3	24.7	8	27.8
	4	1.88	9	15.2	4	65.2	9	12.1
	5	6.25	10	30.5	5	45.2	10	42.9
	1	<0.001	6	2.33	1	5.2	6	22.1
	2	0.131	7	<0.001	2	16.6	7	15.2
7	3	<0.001	8	3.21	3	11.9	8	9.25
	4	0.07	9	1.97	4	12.6	9	8.8
	5	0.03	10	0.06	5	8.1	10	14.2
	1	<0.001	6	0.23	1	4.0	6	11.2
	2	<0.001	7	0.09	2	0.6	7	6.2
14	3	<0.001	8	<0.001	3	4.4	8	3.4
	4	<0.001	9	0.20	4	1.2	9	0.87
	5	<0.001	10	<0.001	5	4.6	10	2.26
	1	<0.001	6	<0.001	1	0.6	6	1.2
	2	<0.001	7	<0.001	2	0.02	7	0.09
21	3	<0.001	8	<0.001	3	1.5	8	0.03
	4	<0.001	9	<0.001	4	2.7	9	<0.001
	5	<0.001	10	<0.001	5	<0.001	10	0.03
	1	<0.001	6	not tested	1	<0.001	6	<0.001
	2	<0.001	7	not tested	2	<0.001	7	<0.001
30	3	<0.001	8	not tested	3	<0.001	8	<0.001
	4	<0.001	9	not tested	4	0.01	9	0.05
	5	<0.001	10	not tested	5	<0.001	10	<0.001

As part of the same study, samples of internal organs and muscle from fish used in a clinical trial were taken after single intramuscular treatment of 20 mg/kg bw (Park *et.al.*, 2013). The fish were maintained at a water temperature range of 21-22 °C.

Mean residue concentrations of 6.0, 2.5, and <0.001 µg/kg were reported at days 5, 7, and 14, respectively, in muscle samples (14 samples per day). Internal organs (combined liver, kidney and spleen) were only sampled at day 5 and a mean residue concentration of 200 µg/kg was measured. For detailed results see Table 6. The authors of the study proposed a withdrawal period of 10 days for use of ampicillin at a dosage of 20 to 40 mg/kg bw via intramuscular injection when the drug is administered at a water temperature of 22 °C or higher.

Table 6. Ampicillin concentrations in samples from muscle and internal organs of flatfish at 5 to 14 days after treatment at a dosage of 20 mg/kg via single intramuscular use (Park *et.al.*, 2013)

Days after administration	Ampicillin concentration (µg/kg)					
	Fish farm 1			Fish farm 2		
	No.	Muscle	Internal organs	No.	Muscle	Internal organs
5	1	3.40	351.90	1	6.40	11.50
	2	5.40	612.80	2	0.88	15.70
	3	8.75	743.90	3	0.35	105.40
	4	10.21	45.20	4	8.80	225.2
	5	5.27	87.29	5	12.39	23.78
	6	4.44	99.40	6	6.27	324.1
	7	5.32	47.20	7	5.67	109.2
7	1	<0.001	Not tested	1	3.80	Not tested
	2	2.40		2	4.83	
	3	2.73		3	4.40	
	4	1.58		4	<0.001	
	5	<0.001		5	<0.001	
	6	<0.001		6	1.0	
	7	1.58		7	0.40	
14	1	<0.001	Not tested	1	<0.001	Not tested
	2	<0.001		2	<0.001	
	3	<0.001		3	<0.001	
	4	<0.001		4	<0.001	
	5	<0.001		5	<0.001	
	6	<0.001		6	<0.001	
	7	<0.001		7	<0.001	

In another study, residue concentrations after single intramuscular injection of ampicillin at doses of 20, 40 and 80 mg/kg bw in flatfish (species not specified, bw 350-550 g) were investigated at a water temperature range of 22 ± 3 °C (Toxicological Research Center. 2014). The flatfish were harvested at days 5, 7, and 10. An LC-MS/MS method was used for analysis

and validation data were provided (LOQ 10 µg/kg). Ampicillin was not detected in any of the samples.

No information on the position of sampling sites in relation to injection sites was provided in the residue depletion studies. However, considering the fish anatomy, the high solubility of ampicillin sodium and the rapid absorption rate, the Committee considered it likely that concentrations in injection sites are similar to those in other muscle tissues.

Summarized results from the residue depletions studies are shown in Table 7.

Table 7. Summary of residue depletion data in fish muscle

Reference	Species	Dose	No. of samples per slaughter day	Water temperature (°C)	Mean residue concentrations in muscle after end of treatment (µg/kg)									
					Day 1	Day 2	Day 3	Day 4	Day 5	Day 7	Day 9	Day 10	Day 13	Day 14
Cho et al., 2006	olive flounder	100 mg/kg bw/day in feed for 5 days	5	25	143 ± 38	52 ± 22	16 ± 6	ND	-	-	-	-	-	-
Cho et al., 2006	rockfish	100 mg/kg bw/day in feed for 5 days	5	25	138 ± 54	45 ± 19	12 ± 4	ND	-	-	-	-	-	-
Cho et al., 2006	red sea bream	100 mg/kg bw/day in feed for 5 days	5	25	187 ± 42	63 ± 28	21 ± 7	ND	-	-	-	-	-	-
Son et al., 2011	olive flounder	20 mg/kg bw/day in feed for 5 days	10	13.4-15.6	41	-	23	-	-	-	-	-	<7 (LOQ)	-
Son et al., 2011	olive flounder	40 mg/kg bw/day in feed for 5 days	10	16.8-21.4	30	-	18	-	-	-	<7 (LOQ)	-	-	-
Marine Science Research Center, 2013	flatfish	40 mg/kg bw/day intramuscular use single treatment	10	22 ± 3	-	35.5 ± 24.6	-	-	10.1 ± 9.2	1.1 ± 1.3	-	-	-	<0.001
Marine Science Research Center, 2013	flatfish	200 mg/kg bw/day intramuscular use single treatment	10	22 ± 3	-	422.7 ± 342.4	-	-	44.6 ± 30.1	12.7 ± 4.7	-	-	-	3.9 ± 3.0
Marine Science Research Center, 2013	flatfish	20 mg/kg bw/day intramuscular use single treatment	14	21-22	-	-	-	-	5.97 ± 3.20	2.52 ± 1.46	-	-	-	<0.001
TR14008, 2014	flatfish	40 mg/kg bw/day intramuscular use single treatment	10	22 ± 3	-	-	-	-	<10 (LOQ)	<10 (LOQ)	-	<10 (LOQ)	-	-
TR14008, 2014	flatfish	80 mg/kg bw/day intramuscular use single treatment	10	22 ± 3	-	-	-	-	<10 (LOQ)	<10 (LOQ)	-	<10 (LOQ)	-	-
TR14008, 2014	flatfish	single treatment	10	22 ± 3	-	-	-	-	<10 (LOQ)	<10 (LOQ)	-	<10 (LOQ)	-	-

Monitoring data in several fish species

In addition, data from a monitoring study on the occurrence of ampicillin in aquaculture products in a member state were available (Anonymous, 2015). A total of 958 saltwater and freshwater fish and crustacean (halibut, flatfish, sea bass, convict grouper, sea bream, Korean bullhead, catfish, loach, croaker, lobster, rockfish, crucian carp, shrimp, trout, gray mullet, salmon, carp, eel, gizzard shad, and leather carp) harvested at aquaculture farms were collected from retail stores and wholesale markets. Samples were taken and analysed for residue concentrations of ampicillin and amoxicillin using an LC-MS/MS (method validated according to CODEX guidelines CAC/GL-16 and CL-71, LOQ 1 µg/kg).

Ampicillin residues were not detected in any of the samples. However, no information on ampicillin treatments was given, and as such, the relevance of the results cannot be assessed.

Methods of analysis for residues in tissues

Several methods for the determination of ampicillin in food, feed and biological matrices have been reported. As this monograph on ampicillin is focused on residues in fish tissues, only analytical methods developed and validated for fish species are described here. The Committee assessed the validation data available for these methods against the analytical requirements as published in CAC/GL71-2009 (FAO/WHO, 2014).

Methods suitable for screening samples for potential non-compliant residues are available. In addition, quantitative methods based on high performance liquid chromatography with tandem mass spectrometry (LC-MS/MS) and high performance liquid chromatography with fluorimetric detection (HPLC/FL) were developed and validated in compliance with GLP.

No information on metabolism of ampicillin in fish is available, but it is considered unlikely that other metabolites than in mammals would occur in fish tissues.

Screening methods

Screening methods are summarized in Table 8.

Bacillus stearothermophilus Disk Assay

A quantitative *Bacillus stearothermophilus* disk assay, which was originally developed for use in milk (AOAC, 1990), was validated for use in muscle of channel catfish and striped bass (Plakas *et.al.*, 1991). Sensitivity of the assay and extraction efficacy were determined in spiked muscle from both species at concentrations from 25 µg/kg up to 1000 mg/kg. Ampicillin was completely recoverable (99-104 %) and the limit of determination was at 25 µg/kg.

Microbiological Assay

Lee *et.al.*, 2007, developed an analytical method for measurement of residues (13 antibiotics including ampicillin) in food, using two microbial assays (to prescreen for possible antibiotic-containing foods) and HPLC. The method was validated for eel (n = 70), flatfish (n = 17), armorclad rockfish (n = 18), sea bream (n = 18), perch (n = 18), and oyster (n = 4). *Bacillus megaterium* ATCC 9885, *Bacillus subtilis* ATCC 6633, *Bacillus cereus* ATCC 11778,

and *Bacillus stearothermophilus* ATCC 10149 were used for a buffer solution assay (BSA) and a direct absorbing assay (DAA). Samples with positive results were analyzed using HPLC with UV and fluorescence detection. Recovery rates for HPLC was 97.8 %, LOD 4 µg/kg and 16 µg/kg.

When the microbial assay screening results were compared with HPLC results, all the HPLC positive samples equal to or above the LOQ were included in the positive results of the direct absorbing method. However, the buffer solution method only detected two samples as positive when compared with the HPLC results. BSA had a greater possibility for false negatives than had DAA. Both microbial assays were not validated according to CAC/GL 71-2009.

Solid phase fluorescence immunoassay (SPFIA) method

A SPFIA method developed for antibiotic residue detection in milk was applied for ampicillin analysis in fish muscle (Jung *et.al.*, 2006). The recovery rates of spiked samples at 10 and 50 µg/kg were 93.4 % and 91.4 %, respectively. The detection limit of ampicillin in muscle of olive flounder was calculated at 5 µg/kg.

Quantitative methods

Single-residue methods

Liquid chromatography (LC)

A single-residue method for detection of ampicillin in animal muscle tissues by high-performance liquid chromatography with fluorescence detection was shown to be suitable also for use in catfish muscle. The analytical method (Luo *et.al.*, 1997) consisted of extraction using 0.01 M phosphate puffer (pH 4.5) followed by precipitation with 75 % trichloroacetic acid and derivatisation of ampicillin from muscle samples (5.0 g) with formaldehyde under acid and heating conditions. For the HPLC-fluorescence analysis of the ampicillin derivative maximum excitation wavelength and maximum emission wavelength of 346 nm and 422 nm were used. The retention time of the ampicillin fluorescent derivative was 12.5 min. The fluorescent derivative was well resolved from interfering peaks. The linearity of the calibration curve for ampicillin was good ($r^2=0.999$) for a range of concentrations equivalent to 1 µg/kg to 50 µg/kg of ampicillin in the muscle tissue samples. Mean within-day recoveries from fortified catfish muscle tissue samples were 93.9 % (2.9 % CV), 89.9 % (4.3 % CV) and 95.2 % (1.8 % CV) for fortification levels of 5 µg/kg, 10 µg/kg and 20 µg/kg respectively. For day-to-day recovery, a fortification level of 10 µg/kg was used, resulting in a mean recovery of 91.4 % (3.6 % CV) for catfish samples. The LOD and LOQ of the analytical method for ampicillin residues were calculated as 0.6 µg/kg and 1.5 µg/kg of muscle tissues, respectively.

Another LC method with UV detection (Nagata and Saeki, 1986) was developed for use in fish muscle. Ampicillin is extracted from tissues with methanol, and the extract is evaporated to dryness with addition of n-propyl alcohol to inhibit bumping. This residue is cleaned up by Florisil cartridge chromatography. LC analysis is carried out on a Nucleosil C18 column, and ampicillin is quantitated by ultraviolet detection at 22 nm. Recoveries of ampicillin added to tissues at levels of 200 µg/kg and 100 µg/kg were 73.2 % and 61.5 %, respectively. The limit of detection was 30 µg/kg.

Table 8. Summary of ampicillin single-residue method for use in fish muscle (LOD = limit of detection, LOQ = limit of quantitation)

Reference	Method	Fish species	LOD (µg/kg)	LOQ (µg/kg)	Recovery (%)	Validation range (µg/kg)
Plakas <i>et.al.</i>, 1991	<i>Bacillus</i> <i>stearothermophilus</i> Disk Assay	Striped bass (<i>Morone saxatilis</i>)	25	-	99-104	25-1000
		Channel catfish (<i>Ictalurus punctatus</i>)				
Jung <i>et.al.</i>, 2006	Solid phase fluorescence immunoassay	Olive flounder (<i>Paralichthys olivaceus</i>)	5	-	91.4-93.4	5-50
Luo <i>et.al.</i>, 1997	HPLC with fluorescence detection	Catfish Species not specified	0.6	1.5	>85 %	5-20
Nagata and Saeki, 1986	HPLC with UV detection	Yellowtail Species not specified	30	-	61.5-63.2	3-150

Multi-residue methods

For analysis of residue concentrations, an LC-MS/MS simultaneous multi-class detection method was used (Korean report). 2.0 g homogenized samples were transferred to a 50 mL polypropylene tube, treated with ammonium formate (10 mL, 2 mM) in 80 % acetonitrile, and subjected to 10 min centrifugation at 10,000 g. The supernatant was separated and poured into the polypropylene tube. A dispersive sorbent (Prep C18, 500 mg) and hexane (10 mL) were sequentially added, and the mixture was thoroughly mixed for 30 s using a vortex mixer, followed by 5-min centrifugation at 10,000 g. The hexane layer was discarded, and 5 mL of the supernatant was transferred to a 15-mL polypropylene tube and concentrated to 1 mL under nitrogen at 40 °C. The extract was filtered, and a 5-µL aliquot was injected into the LC-MS/MS instrument. Liquid chromatography analysis was performed on an ACQUITY UPLC system coupled with a XEVO TQ-S tandem quadrupole mass spectrometer (Waters, Milford, MA, USA). An X-SELECT C18 column (2.1 mm × 150 mm, 3.5 µm, Waters, Dublin, Ireland) was used for separation. The mobile phase consisted of solutions of formic acid (0.1 %) in water (A) and in acetonitrile (B). Chromatographic separation was performed in gradient mode (0–1 min: 0.4 mL/min, 10 % B; 1–6 min: 0.4 mL/min, increase to 40 % B; 6–10 min: 0.4 mL/min, linear increase to 95 % B and 15-min hold, 0.4 mL/min; 15–15.1 min: decrease to 10 % B and 20-min hold, 0.4 mL/min). The column was maintained at 40 °C, and the flow rate was set to 0.4 mL/min. Spray voltages of 5.0 and –3.5 kV were used in positive and negative ESI modes, respectively, and the capillary temperature was set to 350 °C. Multiple reaction monitoring (MRM) were used for target identification, with precursors and three fragment ions listed in Table 9.

Table 9. LC-MS/MS parameters used for quantifying Ampicillin

Analyte	Ionization mode	Molecular weight	Precursor ion (<i>m/z</i>)	Product ion, <i>m/z</i> (CE, eV)
Ampicillin	Positive	349.4	350	106(19), 114(29), 174(11)

Validation of the analytical method was carried out according to CODEX guidelines (CAC/GL-16 and CL-71) to evaluate the performance of the employed analytical method. The validation level of each veterinary drug substance was performed based on Korean MRL (Maximum Residue Limit) for fishery products. Matrix-matched calibration curves were constructed using blank fish samples fortified with the standard solution. Recoveries were determined by spiking blank fish samples at levels of 0.5, 1, and 2 times the MRL, using five replicates for each concentration level in one day. The limits of detection (LOD) and quantification (LOQ) were calculated for signal-to-noise (S/N) ratios of 3 and ten, respectively. Validation data are summarized in Table 10. LOQs were calculated based on minimal acceptable signal to noise (S/N) ratios, being low enough to detect veterinary drugs at levels close to their maximum residue limits (MRLs). Most of the observed recoveries exceeded 60 %, and most CVs were below 15 %. Linearities, expressed as the square of correlation coefficients, were greater than 0.98.

Table 10. Recoveries and coefficients of variation for Ampicillin in flatfish, eel, and shrimp

LOQ (µg/kg)	Spiking (µg/kg)	Flatfish		Eel		Shrimp	
		Recovery (%)	CV (%)	Recovery (%)	CV (%)	Recovery (%)	CV (%)
1	25	63	14	60	8	68	9
	50	75	13	87	6	79	10
	100	90	1	90	2	87	4

Capillary electrophoresis-mass spectrometry

A specific CE-MS method was developed for the simultaneous determination of 12 antibacterial residues (four sulfonamides: sulfamethazine, sulfathiazole, sulfadiazine, and sulfachlorpyridazine; four β -lactams: amoxicillin, ampicillin, oxacillin, and penicillin V, and four quinolones: danofloxacin, enrofloxacin, ofloxacin, and flumequine) in fish and livestock (Juan-Garcia *et.al.*, 2007). Separation conditions, sheath liquid composition and electrospray parameters were optimized to obtain adequate CE separation and a high sensitivity. CE employed a 75 cm long fused-silica capillary (50 cm thermostated plus 25 cm at room temperature) 75 µm id and a 60 mM ammonium acetate separation buffer at pH 8 with 10 % of methanol. The minimum number of identification points, according to the 2002/657/EC European Decision, was achieved using an IT in multiple reaction monitoring (MRM) mode.

For quantification in meat and fish samples, a two-step procedure was developed using ACN to extract the antibacterials and to precipitate the proteins and dispersive SPE, with C18 to clean up the extract. External matrix matched calibration curves were used to achieve accurate quantitative results. The limit of detection and limit of quantification for ampicillin in muscle of gilthead bream (*Sparus aurata*) were 5 and 20 µg/kg respectively.

Liquid chromatography (LC)

A confirmatory high performance liquid chromatography method for the determination of six penicillin antibiotics (ampicillin, penicillin G, penicillin V, oxacillin, cloxacillin, dicloxacillin) and three amphenicol antibiotics (thiamphenicol, florfenicol, chloramphenicol) in gilthead seabream (*Sparus Aurata*) tissue is available (Evaggelopoulou and Samanidou, 2013). Substances were separated on an Inertsil, C8 (250 x 4 mm, 5 µm) column by gradient elution with a mobile phase consisting of ammonium acetate 0.05 M and acetonitrile at 25 °C. Antibiotics were isolated from gilthead seabream tissue by liquid–liquid extraction and further clean-up was performed by solid phase extraction using Oasis HLB (200 mg/6 mL) cartridges. The developed method was fully validated in terms of selectivity, linearity, accuracy, precision, stability and sensitivity according to the European Union Decision 2002/657/EC. The limit of detection and limit of quantification for ampicillin in muscle of gilthead bream (*Sparus aurata*) were 11.6 and 35.2 µg/kg respectively.

HPLC with diode array and fluorescence detection in combination with enzymatic probe sonication extraction was developed for 11 antibiotic substances and the same species as above (Fernandez-Torres *et.al.*, 2010). Ampicillin and its main metabolites ampicilloic acid are measured. The method is simple and provides good validation parameters in terms of linearity, precision. The recovery rate of ampicillin in muscle of anchovy, wedge sole and hake were higher than 59 %. Instrumental LOD and LOQ were 0.12 and 0.56 µg/mL, leading to method LOQ and LOD higher than 0.09 and 0.43 µg/g for the three fish species (see Table 11).

Liquid chromatography – mass spectrometry (LC-MS)

A LC method developed for 11 antibiotics (including ampicillin) uses enzymatic-microwave assisted extraction prior to high performance liquid chromatography-mass spectrometry (Fernandez-Torres *et.al.*, 2011a). The main factors affecting the extraction efficiency were optimized in tissue of hake (*Merluccius merluccius*), anchovy (*Engraulis encrasicolus*), mussel (*Mytilus* sp.) and wedge sole (*Solea solea*). The separation of the analysed compounds was conducted by means of a Phenomenex® Gemini C18 (150 mm×4.6 mm I.D., particle size 5 µm) analytical column with LiChroCART® LiChrospher® C18 (4 mm×4 mm, particle size 5 µm) guard-column. Analysed drugs were determined using formic acid 0.1 % in water and acetonitrile in gradient elution mode as mobile phase. The method was validated checking the performance parameters of the analytical method as indicated in the Commission Decision 2002/657/EC. The limit of detection and limit of quantification for ampicillin in muscle of fish were 2 and 9 µg/kg respectively. The recovery rates at 50, 100 and 200 µg/kg were higher than 85 % for fish species.

An analytical method for the determination of 32 veterinary drug residues belonging to several families in gilthead sea bream (*Sparus aurata*) by ultra high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC–MS/MS) was developed by Lopes *et.al.*, (2012). The extraction was based on modified QuEChERS (quick, easy, cheap, effective, rugged and safe) procedure, using as extraction solution a mixture of acetonitrile and methanol (75:25, v/v). The method was validated according to the European Union Decision 2002/657/EC. The LOD and LOQ were 3 and 10 µg/kg. Precision in terms of relative standard deviation (RSD) was under 19 % for ampicillin, and the recoveries ranged from 93 % to 118 %.

Another method for the determination of 41 antibiotics from seven different classes in gilthead seabream (*Sparus aurata*) by ultra-high-performance liquid chromatography with tandem mass spectrometry (UPLC-MS/MS) is available (Freitas *et.al.*, 2014). Fourteen procedures for sample treatment were tested and an extraction with acetonitrile and ethylenediaminetetra acetic acid was found to be the best option. The methodology was validated in accordance with Decision 2002/657/EC. Precision in terms of relative standard deviation (RSD) was under 13 % for ampicillin, and the recovery was 104 %.

Smith *et.al.*, (2009) established a method based on LC-ion trap mass spectrometry to screen and confirm 38 compounds from different classes of drugs (including ampicillin) for use in trout, salmon, catfish and tilapia. Samples were extracted with acetonitrile and hexane. The acetonitrile phase was evaporated, redissolved in water and acetonitrile, and analyzed by gradient chromatography on a phenyl column. MS2 or MS3 spectra were monitored for each compound. Qualitative method performance was evaluated by the analysis over several days of replicate samples of control fish, fish fortified with a drug mixture at 1 ppm, 0.1ppm and 0.01 ppm, and fish dosed with a representative from each drug class. The method was validated.

A quantitative screening method using high-resolution liquid chromatography accurate mass time-of-flight mass spectrometry was developed for detection of about 100 veterinary drugs (including ampicillin) in fish tissues and validated according to EU Commission Decision 2002/657/EC (Peters *et.al.*, 2009).

A rapid multi-residue/multi-class procedure utilizing ultra-performance liquid chromatography tandem mass spectrometry (UPLC/MS/MS) detection has been developed and validated to monitor 18 veterinary drugs, including ampicillin (Tang *et.al.*, 2012). Two grams of homogenized fish muscle sample (species not specified) were fortified with the 18 drugs. The drugs were extracted using two extraction solutions and then were cleaned up with n-hexane and SPE. Data acquisition with MS/ MS was achieved using the multiple reaction monitoring modes to acquire the data. The method was validated at four concentration levels (50, 100, 150, and 200 mg/kg), with average recovery of ampicillin in fish muscle at 102.6 % and inter-day and intra-day coefficients of variation (CV) values at 10.6 % and 6.2 %, respectively.

Table 11. Summary of ampicillin multi-residue methods. All methods listed, except Fernandez-Torres *et.al.*, (2010) and Smith *et.al.*, (2009), were validated according to EU Commission Decision 2002/657/EC.

Ref.	Method	Substances	Matrices/ Species	Ampicillin	
				LOD/LOQ	Recovery
Freitas <i>et.al.</i> , 2014	Ultra-high performance liquid chromatography with tandem mass spectrometry	41 antibiotics from 7 classes including ampicillin	Muscle/ Gilthead sea bream	CC _α 64.9 µg/kg CC _β 79.7 µg/kg	104%
Evangelopoulou, 2012	High performance liquid chromatography method	6 penicillin antibiotics including ampicillin 3 amphenicol antibiotics	Muscle + skin/ Gilthead sea bream	LOD 11.6 µg/kg LOQ 35.2 µg/kg	Within day: 97.5-99.4% Between day: 97.2-101.6%
Lopes <i>et.al.</i> , 2012	Ultra high performance liquid chromatography coupled to tandem mass spectrometry	32 veterinary drugs including ampicillin	Muscle + skin/ Gilthead sea bream	LOD 3.0 µg/kg LOQ 10.0 µg/kg	93-106%
Juan-García <i>et.al.</i> , 2007	Capillary electrophoresis-mass spectrometry	4 sulfonamides 4 beta-lactams including ampicillin 4 quinolones	Muscle/ Gilthead sea bream	LOD 5 µg/kg LOQ 20 µg/kg	74-80%
Fernández-Torres <i>et.al.</i> , 2011b	Reversed-phase High-Performance Liquid Chromatography with Diode Array-Fluorescence (HPLC-DAD-FLD) Detection	11 antibiotics including ampicillin	Muscle/ Hake Wedge sole	LOD 120 µg/kg LOQ 410-420 µg/kg	61.9-69.6%
Fernández-Torres <i>et.al.</i> , 2011a	Enzymatic-microwave assisted extraction and high-performance liquid chromatography–mass spectrometry	11 antibiotics including ampicillin	Muscle/ Hake Anchovy Wedge sole	LOD 5 µg/kg LOQ 15-17 µg/kg	70-74% 82-84% 73-82%

Smith <i>et al.</i> , 2009	Liquid chromatography-ion trap mass spectrometry	38 compounds: quinolones, fluoroquinolones, sulfonamides, β -lactams (including ampicillin), tetracyclines, lincosamides, ionophores, macrolides, dyes	Muscle + skin/ Rainbow Trout Atlantic Salmon Channel Catfish Tilapia	LOQ 100 $\mu\text{g/kg}$ LOQ 100 $\mu\text{g/kg}$ LOQ 100 $\mu\text{g/kg}$ LOQ 1000 $\mu\text{g/kg}$	<50%
Peters <i>et al.</i> , 2009	High-resolution liquid chromatography accurate mass time-of-flight mass spectrometry	100 veterinary drugs including ampicillin	Muscle/ Salmon Trout Mackerel Eel Catfish	CC _{β} 155–156 $\mu\text{g/kg}$	100-112%
Tang <i>et al.</i> , 2012	Ultra performance liquid chromatography tandem mass spectrometry	18 veterinary drugs including ampicillin	Muscle/ Fish species not specified	LOD 0.5 $\mu\text{g/kg}$ LOQ 1 $\mu\text{g/kg}$	102.6%

Stability of residues

The stability of ampicillin in fish muscle was not described but it was demonstrated that ampicillin is stable in pig muscle during 308 and 360 days when stored at -18 °C and -70 °C respectively. Solution of ampicillin in a mixture ethanol/water 50/50, v/v is stable only 1 month when stored at -18 °C (Gaugain *et al.*, 2013).

Appraisal

Ampicillin has not been previously been evaluated by the Committee. Ampicillin is a β -lactam antimicrobial agent with a bactericidal mode of action. It is registered for aquaculture use in the treatment flat fish (unspecified yellowtail, olive flounder) as an oral powder via feed at a daily dose of 5 to 20 mg/ kg bw for five days respectively via intramuscular injection at a single dose of 20 mg/kg bw.

No residue depletion studies using radiolabeled ampicillin in fish were available for evaluation. Four residue depletion studies using unlabeled ampicillin (two using oral application and two using intramuscular treatment) showed fast and continuous decline of residues from muscle tissues. Ampicillin is considered to be the relevant marker residue.

The available data on ampicillin are not sufficient to establish an MRL for finfish.

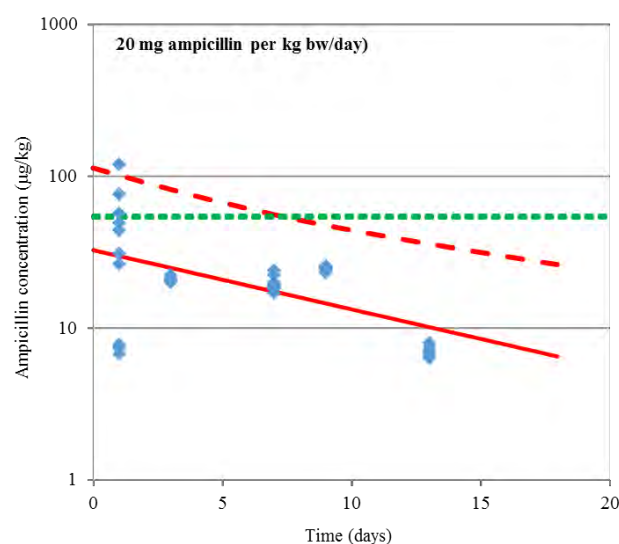
The recommended MRL of 50 $\mu\text{g/kg}$ for ampicillin in finfish muscle and muscle and skin in natural proportions was derived from those established for amoxicillin. To do this recommendation, the committee has considered the similarity of physico-chemical properties,

antibacterial activities, the toxicological profiles as well as the pharmacokinetic profiles between ampicillin and amoxicillin.

Qualitative and quantitative single and multi-residue methods are available to determine residues of ampicillin, the microbiologically active residue identified in fish muscle and in muscle and skin in natural proportions. Validated analytical methods, HPLC-UV and LC-MS/MS were reviewed. LC-MS/MS methods are appropriate for screening, quantification and monitoring of ampicillin residues in fish tissues.

Residue depletion data from the study conducted in olive flounder after oral use of 20 mg/kg bw for five consecutive days (Son *et.al.*, 2011) were used to derive mean values and 95/95 upper tolerance limits for exposure calculation (0).

Figure 6. Residue depletion curves of ampicillin in olive flounder based on data by Son *et.al.*, (2011), oral use of 20 mg/kg bw for five consecutive days



Dietary Exposure

Dietary exposure to ampicillin residues from food is considered to occur only from its use as a veterinary drug because there are no CODEX MRLs for ampicillin. Based on the hazard profile of the compound, acute dietary exposure is required for the general population and children. Chronic exposure estimates for children and shorter than lifetime dietary exposure are not required. Dietary exposure was estimated based on the potential occurrence of ampicillin residues in a single food (finfish muscle and skin in natural proportion) after 5 days of treatment (73 degree-days). Consumption inputs were based on the highest reported values available within the fish category (i.e., marine fish consumptions for means, freshwater fish consumption for the 97.5th percentile).

Chronic 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 of 0–3 µg/kg bw set by JECFA during this meeting (Table 12).

In addition to the accepted GECDE methodology, further calculations were carried out. Instead of using the highest mean and the highest 97.5th percentile consumption across surveys (Table 13), the calculations were carried out using the mean and the highest reliable percentile for each individual national survey from available data sets (CIFOCCOs). The mean and ranges across surveys were reported. The mean of 25 estimates for adults or the general population was 0.05 µg/kg bw per day (2 % of the upper bound of the ADI), with a range of 0.001–0.29 (<1–10 % of the upper bound of the ADI).

Acute Exposure

The GEADE for the general population is 1.9 µg/kg bw per day, which represents 16 % of the ARfD of 12 µg/kg bw (Table 14). The GEADE for children is 1.7 µg/kg bw per day, which represents 14 % of the ARfD. For both population groups, finfish muscle and skin in natural proportion was the only contributor to acute dietary exposure.

Table 12. Estimated chronic dietary exposure to ampicillin (GECDE) occurring in finfish muscle

Category	Type	Median concentration ¹ n ¹ (µg/kg)	Mean consumption ² (whole population, g/kg bw/day)	Highest reliable percentile consumption ³ (consumers only, g/kg bw) / [percentile used]	MR:TR ratio	Exposure (µg/kg bw/day) mean 97.5th	GECDE ⁴ µg/kg bw/day	ADI %
General Population								
Fish and seafood	Finfish ⁵	21	0.4	13.6 [97.5]	1	0.008 0.29		
TOTAL						- 0.29 0.29		10

¹Median concentration after 5 days of treatment (73 DD)
²highest mean consumption figures based on whole population considered from the available dataset
³highest food consumption figures based on consumers only considered from the available dataset
⁴GECDE is the sum of the highest exposure at the highest reliable percentile of consumption for a food and the mean dietary exposures of the other foods
⁵muscle and skin in natural proportion

Table 13. Estimated chronic dietary exposure to ampicillin (GECDE) occurring in finfish muscle

Country	Population group	Survey	GECDE ¹ (µg/kg bw per day)	%A DI	Major contributing food ²
Bangladesh	Adult women	Harvest_2007/8	0.038	1	Freshwater fish
Belgium	Adults	Diet_National_2004	0.044	2	Cod
Brazil	General population	Brazilian Institute of Geography and Statistics	0.289	10	Freshwater fish
Burkina Faso	Adult women	Harvest_2010	0.001	<1	Freshwater fish
China	General population	2002 China Nutrition and Health Survey	0.102	3	Freshwater fish
Czech Republic	Adults	SISP04	0.032	1	Herring
Denmark	Adults	Danish Dietary Survey	0.015	1	Plaice
Finland	Adults	FINDIET_2007	0.056	2	Pacific salmon
France	Adults	INCA2	0.023	1	Cod
Germany	Adults	National Nutrition Survey_II	0.053	2	Herring
Hungary	Adults	National Repr_Surv	0.039	1	Carps
Ireland	Adults	NSIFCS	0.027	1	Pacific salmon
Italy	Adults	INRAN_SCAI_2005_06	0.042	1	Cod
Japan	General population	DSFFQ_FI	0.022	1	Marine fish
Latvia	Adults	EFSA_TEST	0.025	1	Herring

Country	Population group	Survey	GECDE ¹ (µg/kg bw per day)	%A DI	Major contributing food ²
Netherlands	Adults	DNFCS_2003	0.014	1	Pacific salmon
South Korea	General population	KNHNES	0.111	4	Pollack
Spain	Adults	AESAN	0.054	2	Marine fish
Spain	Adults	AESAN_FIAB	0.060	2	Marine fish
Sweden	Adults	Riksmaten_1997_98	0.010	<1	Herring
Thailand	General population	FCDT	0.019	1	Freshwater fish
Uganda	Adult women	Harvest_2007	0.031	1	Freshwater fish
United Kingdom	Adults	NDNS	0.027	1	Pacific salmon
USA	Childbearing women	What We Eat in America - Food Commodity Intake Database	0.058	2	Freshwater fish
USA	General Population	What We Eat in America - Food Commodity Intake Database	0.059	2	Freshwater fish
		Mean	0.050	2	
		Minimum	0.001	<1	
		Maximum	0.289	10	

¹ GECDE is the sum of the highest exposure at the highest reliable percentile of consumption for a food and the mean dietary exposures of the other foods

²muscle and skin in natural proportion

Table 14. Estimated acute dietary exposure to ampicillin (GEADE) occurring in finfish muscle

Category	Type	95 th centile concentration ¹ (µg/kg)	Acute Consumption ² (g/kg bw)	MR:TR ratio	GEADE ³ mg/kg bw/day	ARfD %
General Population						
Fish and seafood	Finfish ⁴	67	27.8	1	1.9	16
Children						
Fish and seafood	Finfish ⁴	67	24.9	1	1.7	14

¹95/95 UTL concentration after 5 days of treatment (73 DD)
²highest food consumption figures based on the 97.5th percentile consumption from available data set
³GEADE is the product of the 97.5th level of consumption and the highest residue
⁴muscle and skin in natural proportion

Maximum Residue Limits

In order to address the ambiguity of the terms 'flat fish' and 'finfish', the Committee decided that for the purpose of this report, the term finfish will include all fish species. In recommending MRLs for ampicillin in finfish, the Committee considered the following factors:

An ADI of 0-0.003 mg/kg bw for ampicillin was established by the Committee based on a microbiological endpoint. The Committee concluded that for ampicillin there are no specific concerns for less-than-lifetime exposure.

- An ARfD was established at 0.012 mg/kg bw for ampicillin based on a microbiological endpoint.
- Ampicillin is authorized for use in at least two fish species in one member state. The withdrawal periods of approved uses are unknown. Only withdrawal periods as proposed by the authors of the residue studies are available.
- Ampicillin is not lipophilic, not widely distributed in fish tissues and tissues residues deplete rapidly.
- Parent ampicillin is of relevance for the microbiological potential of the compound.
- No metabolism data in fish were available.
- Ampicillin is the relevant marker residue.
- Some residue data in flat fish muscle were available. However, they did not meet the data and validation requirements for residue studies.
- No residue data in fish skin were available.
- Suitable validated routine analytical methods are available for monitoring purposes and can be applied for tissues of different fish species.
- The available data on ampicillin are not sufficient to establish an MRL for finfish. However, as the modes of action, the physico-chemical properties, the toxicological profiles as well as the pharmacokinetic profiles, are very similar between amoxicillin and ampicillin, the Committee recommends to establish the same MRLs for finfish for both substances.
- Based on the limited data available, median and the 95/95 UTL were calculated. These were suitable to be used as inputs to estimate chronic and acute exposure to ampicillin from finfish.
- The Committee recommended an MRL of 50 µg/kg for ampicillin in finfish muscle and muscle and skin in natural proportions.

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Ethion

First draft prepared by

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and

Alan Chicoine, Saskatoon, Canada

Identity

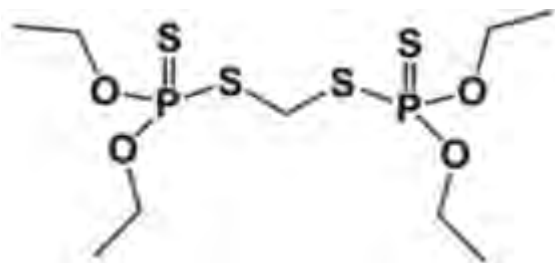
International Non-proprietary Name (INN): Ethion

Synonyms: Diethion; [(Dethoxyphosphinothioylthio) methylthio]-diethoxythioxophosphorane

IUPAC name: O,O,O',O'-Tetraethyl S,S'-methylene bis(phosphorodithioate)

Chemical Abstract Service (CAS) No.: 563-12-2

Structural formula:



Molecular formula: C₉H₂₂O₄P₂S₄

Molecular weight: 384.48 g/mol

Other information on identity and properties

Pure active ingredient: Ethion

Appearance: Colourless to amber-coloured, odourless liquid.

Melting point: -12.2 °C (10.0 °F; 260.9 K)

Solubility (at 20 °C):

Water: 0.0001 %

Vapour pressure: 0.0000015 mmHg (20 °C)

Background

Ethion was previously evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) in 1968, 1972, 1986 and 1990 (JMPR, 1990). Ethion was included for review by the 85th meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) at the request of the Twenty-third Session of the Codex Committee on Residues of Veterinary Drugs in Foods (CCRVDF; FAO/WHO, 2016), to be evaluated using any relevant published data as well as sponsor-submitted residue depletion data. The request was specifically in relation to setting MRLs in edible tissues of cattle.

Residues in food and their evaluation

Conditions of use

Ethion is an organophosphate insecticide used in the prevention of vector-borne diseases carried by the cattle tick, *Boophilus microplus*. It can be formulated into immersion bath treatments, pour-ons, sprays and ear-tags, often in combination with cypermethrin (a pyrethroid insecticide), for administration to cattle (both beef and dairy, depending on the product).

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

Pour-ons also come in solutions, containing (e.g.) 150 g/l ethion and 50 g/l cypermethrin. Recommended doses are 5 ml for animals weighing 100 – 200 kg, 10 ml for animals weighing 200 - 400 kg and 20 ml for animals that weigh >400 kg (3.75 – 7.5 mg/kg).

Ear-tags can contain 36 – 40 g ethion per ear-tag and these are left on the animals for a period of time (e.g. 120 days) until removal. Some products recommend using one ear-tag per animal, some recommend using two.

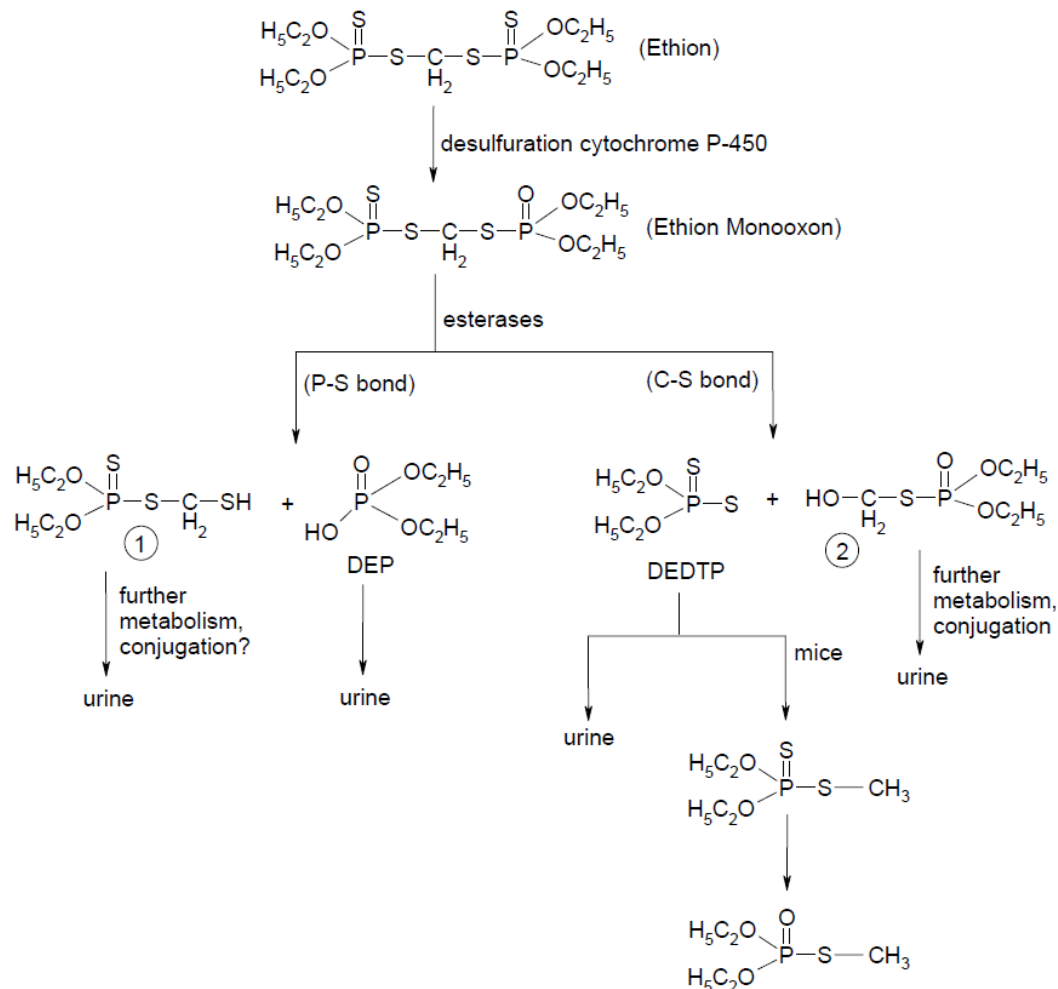
Withdrawal periods for the approved ethion formulations vary from 15 – 45 days, depending on the specific formulation and jurisdiction.

Pharmacokinetics and metabolism

Ethion is a small, lipid-soluble molecule that can be absorbed by passive diffusion through the lungs, gastrointestinal tract, or skin. Absorption appears to be rapid by the oral and dermal routes (depending on species); the time course of absorption is inferred from the onset of clinical signs within 1 hour after accidental ingestion of ethion in a 6-month-old boy (Comstock *et.al.*, 1967) and deaths within 3–6 h in dermally exposed Sherman rats (Gaines 1969). Ethion is desulfurated by cytochrome P450 enzymes in the liver to its active form, ethion monoxon, which causes toxicity due to its potent inhibition of neural acetylcholinesterase.

Ethion and its oxon form can be detoxified by the action of esterases in the blood and liver, producing diethyl phosphate, diethyl thiophosphate, diethyl dithiophosphate, and other metabolites that have not been characterised.

Figure 1. Proposed Mammalian Metabolic Pathways for Ethion (ATSDR, 2000)



DEP = diethylphosphate;

DEDTP = diethyldithiophosphate;

1 = O,O-diethyl-S-mercaptopethylthiophosphate; 2 = O,O-diethyl-S-hydroxymethylthiophosphate

Pharmacokinetics in laboratory animals

Oral administration

Seven days after rats received a single gavage dose of radiolabelled ethion, less than 1 % of the radiolabel was detected in the body (blood, brain, heart, pancreas, leg muscle, lungs, adipose, spleen, bone, skin, hair, kidney, liver, gonads [uterus and ovaries for females, testes, seminal vesicle, and prostate for males]) (Selim 1985a, referenced in ATSDR, 2000). Total residues ranged from 0.21 to 0.34 % of the original dose for females; and 0.18–0.28 % for males. Similar results were obtained in a study where the radioactive dose was given after 14 consecutive daily doses of unlabelled ethion (Selim 1985a).

Elimination from the body is mainly through excretion of water-soluble metabolites in the urine. Conjugation may occur; this is inferred from experiments where [^{14}C -methylene]ethion was administered orally to rats and the radioactivity in urine analysed (Selim 1985b). Samples were extracted with ethyl acetate; the aqueous and organic phases were analysed by high-performance liquid chromatography. More than 99 % of the urine radioactivity was in the aqueous phase. Another sample was acidified (presumably to hydrolyse conjugates) and also extracted with ethyl acetate.

Acidification converted about 30 % of the radioactivity in the aqueous phase to an organosoluble form, which may indicate that some of the products of ethion metabolism are present in urine as conjugates. Four to six radiolabelled metabolites were detected by HPLC, none migrated with standards for ethion, ethion monoxon, or ethion dioxon. None of the metabolites were specifically identified.

Pharmacokinetics in Food-producing Animals

No data are available for cattle. One paper (Mosha *et.al.*, 1990a) was provided that investigated the distribution and elimination of ethion in laying hens and their eggs, another two papers were provided that looked into the distribution into goat tissues and milk (Mosha *et.al.*, 1990b and Mosha *et.al.*, 1991).

Laying Hens

Ten Rhode Island Red laying hens, aged 24-26 weeks and weighing between 1.5-2.1 kg were used in the study (Mosha *et.al.*, 1990a). The birds were housed in two cages and were provided with water and feed *ad libitum*. (^{14}C -methylene)ethion with a specific activity of 2 $\mu\text{Ci}/\text{mg}$ was dissolved in glycerol formal and administered orally to each bird at the dose of 5 mg/kg.

Eggs were collected from the cages in the morning and afternoon. They were individually separated into egg white and yolk, each of which was liquidised. Faeces were collected daily and oven-dried.

Two hens were killed on each of days 1, 3, 7, 15 and 21 after dosing and samples of liver, kidney, skeletal muscle (pectoral muscle), heart, brain and abdominal fat were collected. Blood was collected from each of the two birds at the time of slaughter and on days 5 and 10 from the other hens as pooled samples. All tissues and plasma separated from blood were stored at $-20\text{ }^{\circ}\text{C}$.

The concentration of unchanged ethion in egg-white, yolk and plasma was measured by gas chromatography ("cold" residues), and the concentration of ^{14}C -ethion (ethion and metabolites expressed as ethion equivalents) was determined in tissues, plasma, egg white, yolk and faeces via liquid scintillation counting.

Ethion was extracted from the plasma or egg-white by an equal volume of hexane containing parathion as internal standard. The hexane extract from each egg white was concentrated five times and then analysed using gas chromatography with a nitrogen phosphorus detector (GC-NPD). Recovery rate for ethion in plasma was $94 \pm 5\%$ and $90 \pm 2\%$ in egg-white. The recovery rate from the egg yolk was $69 \pm 4\%$. The detection limit for ethion was 3 $\mu\text{g}/\text{kg}$.

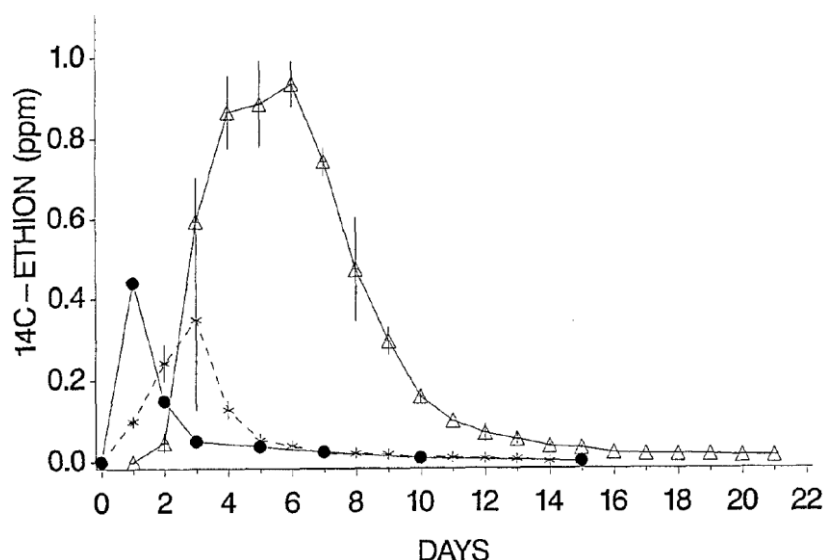
The total amount of ^{14}C -ethion excreted in faeces from the hens was 69 % of the dose. Most of this (58 %) was recovered within 24 h.

The Committee highlighted that ethion residues in laying hens are highest in liver and kidney (Table 1). This contrasts with the results from topically-applied ethion in cattle studies, where the highest concentrations are found in fat (peri-renal and subcutaneous). As the study in hens used radiolabelled ethion, and measured ethion equivalents, it may indicate that it is metabolites of ethion that are being measured in the liver and kidney of the hens. The results for eggs indicate that ethion and its metabolites are preferentially found in more fatty matrices (i.e. the yolk, as opposed to the egg white; Figure 2).

Table 1. Concentration of ^{14}C -ethion in tissues of laying hens

[illegible]

Figure 2. Average concentration of ^{14}C -ethion in plasma, egg white and yolk (from Mosha 1990a).



Legend: black circle = plasma; asterisk = egg white; triangle = yolk.

Goats.

Toxicokinetic parameters and cumulative excretion were studied in goats after intravenous, oral and dermal administration of unlabelled and ^{14}C -ethion (Mosha, 1990b). All goats weighed between 41 and 56 kg and were fed hay and concentrate and given water ad libitum. In the intravenous experiment, six female adult goats (four lactating) were administered 2 mg/kg ^{14}C -ethion by IV infusion. The oral ethion experiment used five lactating goats (dose of 10 mg/kg, three were dosed with ^{14}C -ethion while two received unlabelled ethion). The dermal experiment used four goats (3 lactating) and administered unlabelled ethion (17 % solution) on the skin at the back at 100 mg/kg. The areas of application were clipped before the diluted emulsifiable concentrate was administered. The application site was about 600-700 cm².

Blood samples were obtained just before and at 2 (intravenously only), 5, 10, 15, 20, 30, 45, 60 and 90 min. and at 2, 3, 4, 6, 8, 10, 12, 15, 24, 30 and 48 h, and then once per day until day 14 after exposure. All plasma samples were stored at -20 °C until analysed.

The udder was emptied of milk just before administration, and at 1, 4, 8, 12, 15, 24, 30 and 48 h and thereafter once daily until day 14 post exposure. All milk samples were stored at -20 °C until analysed.

Urine was collected only in those experiments where the radiolabelled drug was administered, as pilot experiments showed that urine did not contain unchanged ethion. The urine was collected quantitatively during the first 48 h after dosing by means of a balloon catheter. After 48 h, all urine was collected from a metabolic cage in which the animal was placed for the 14 days of the experiment. All urine samples were stored at -20 °C before analysis.

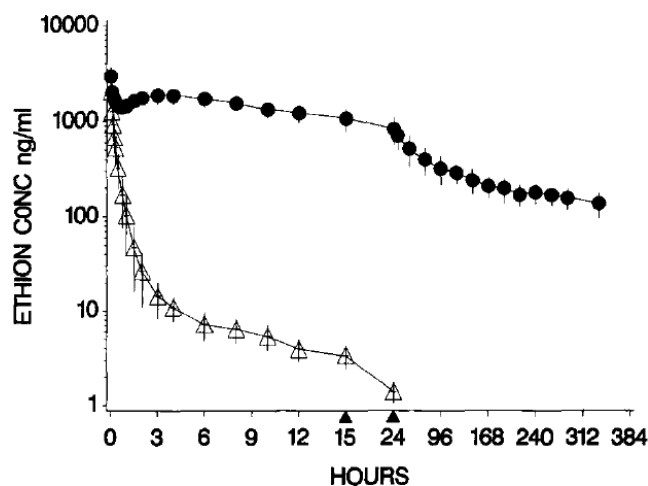
In experiments with ^{14}C -ethion, faeces were collected quantitatively throughout the 14 days and after drying ground into a fine powder.

The quantification of unchanged ethion in plasma and milk was done by gas chromatography. Ethion was extracted from the plasma by an equal volume of hexane containing parathion as an internal standard. Some hexane extracts were concentrated so that ethion in low levels could be quantified. The hexane extracts were analysed on a gas chromatograph with a nitrogen phosphorus detector (NPD). Recovery rate for ethion in plasma was $94 \pm 5\%$. The recovery rate from milk was $101 \pm 4\%$. Detection limit for ethion was $3\text{ }\mu\text{g/kg}$.

The concentration of ^{14}C -ethion (ethion and metabolites) in plasma, milk, urine and faeces was determined by liquid scintillation counting. Tissue and faecal samples for scintillation counting were processed in triplicate.

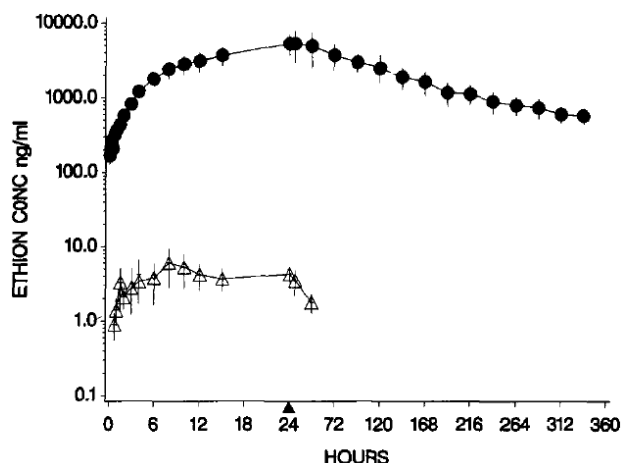
The results IV injection studies (Figure 3) showed an effective half-life ($t_{1/2}$) for unchanged ethion of 2 h, a total body clearance (CL_t) of 3.2 L/kg/h and a volume of distribution (Vd_{ss}) of 9.4 L/kg . Plasma levels of ^{14}C -ethion (ethion + metabolites) were much higher than those of unchanged ethion, and persisted at high levels for more than 14 days. The AUC for ^{14}C -ethion after IV administration was $111 \pm 29\text{ }\mu\text{g.h/ml}$. Cumulative excretion of ^{14}C -ethion was 78 % of the dose with 66 % in urine, 8 % in faeces and 4 % in milk. The much higher and more persistent plasma concentrations of ^{14}C -ethion compared to unchanged ethion (AUC 150 times higher) indicate that elimination of unchanged ethion is mainly due to metabolism. The increase in radioactivity in plasma after an initial decline in the first hour may be attributed to reabsorption from the gastrointestinal tract during enterohepatic circulation.

Figure 3. Mean plasma concentrations vs time for unchanged and ^{14}C -ethion after 2 mg/kg IV administration in goats (from Mosha 1990b).



Legend: Solid circles = total ^{14}C -ethion equivalents, open triangles = unchanged ethion

Figure 4. Mean plasma concentrations vs time for unchanged and ^{14}C -ethion after 10 mg/kg oral administration in goats (from Mosha 1990b).

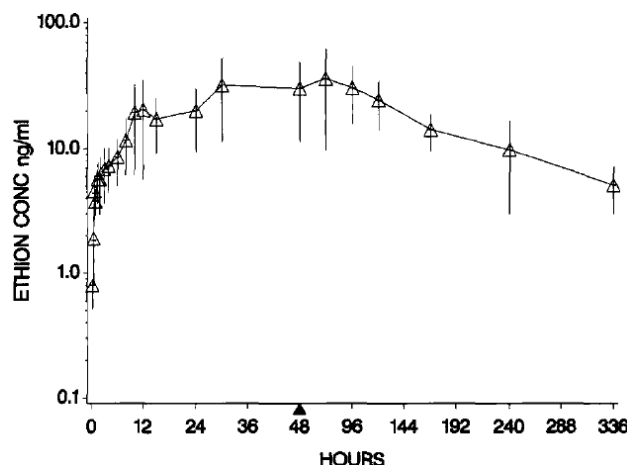


Legend: Solid circles = total ^{14}C -ethion equivalents, open triangles = unchanged ethion

After oral administration (Figure 4), the unchanged ethion levels in plasma were very low and hardly detectable after 48 h. The ^{14}C -ethion concentrations in plasma were more than 100 times higher than unchanged ethion, and persisted in plasma beyond the 14 days sampling period. Less than 5 % of the orally-administered ethion was absorbed unchanged. However, the AUC for ^{14}C -ethion was 5000 times the AUC for unchanged ethion, and proportional to the ^{14}C -ethion AUC obtained after intravenous administration. It is therefore likely that most ^{14}C from orally-administered ^{14}C -ethion is absorbed in goats (though not remaining as parent ethion). The plasma radioactivity had peak values after approximately 30 h. This prolonged absorption of ^{14}C -ethion is probably due to delayed absorption from the rumen, as oral administration of ^{14}C -ethion to rats resulted in a t_{max} of 6 h (JMPR, 1986). Cumulative excretion was 80 % of the dose; with 64 % in urine, 14 % in faeces and 1.7 % in milk.

After dermal administration (Figure 5), unchanged ethion concentrations were higher than after oral application. Dermally-applied ethion concentrations persisted beyond the 14 days sampling period, indicating a prolonged absorption phase. This prolonged absorption period corresponds with the assumption that ethion stays in the epidermis of the skin for a very long time. Approximately 20 % of the dose was absorbed during the 14 days observation period. Only 0.05 % of the dose was excreted unchanged in milk.

Figure 5. The concentration-time profile for unchanged ethion in goat plasma after dermal application of 100 mg/kg (n = 4) (from Mosha 1990b).



The combined recovery of ^{14}C -ethion in urine and milk amounted to 70 and 66 % of the dose after intravenous and oral administration, respectively, indicating an almost complete absorption of ^{14}C -ethion after oral administration. The proportions of the dose excreted in milk were quite small, especially after oral and dermal application. However, the long absorption period after dermal application is responsible for ethion residues found in milk up to five weeks after application. The remaining approximately 20 % ^{14}C -ethion not accounted for probably remains in the tissues where the ^{14}C might have entered the carbon pool to be incorporated in tissue components. The very long terminal elimination phase for ^{14}C -ethion supports this assumption. Collection of exhaled air from three of the goats administered ^{14}C -ethion IV showed that only insignificant amounts of ^{14}C are excreted by this route.

The Committee noted that the results from this study in goats are highly relevant to the current assessment in cattle. This study demonstrates that ethion undergoes extensive metabolism in goats, and that the majority of the ethion-derived residues in cattle are therefore predicted to be metabolites rather than parent ethion.

Ethion residues in goat milk were evaluated in Mosha, 1991 using the same dosage regimen as Mosha, 1990b. Intravenous ethion injection resulted in ^{14}C residues persisting much longer than unchanged ethion in milk. After oral administration, ethion was almost completely metabolised before absorption, leading to very low levels of parent ethion but high levels of ^{14}C in milk. Dermal application of ethion resulted in limited but very prolonged absorption, and detectable residue of ethion in milk for up to 5 weeks.

The Committee noted that this study confirmed the persistence of ethion metabolites in milk (relative to parent ethion). This study confirms that ethion metabolites persist much longer than the parent compound, and that these metabolites were transferred into milk.

Metabolism in Laboratory Animals

Rats

Previously available data (ATSDR, 2000) were reviewed. No new data were available to the Committee.

Metabolism in Food Producing Animals

No data was provided to the Committee, or available from the published literature, to allow characterisation of the metabolic pathway for ethion in cattle.

Comparative Metabolism

Since the data are not available for cattle, no comparison with other species can be made at this time.

Tissue residue depletion studies

Radiolabelled residue depletion studies

No radiolabelled residues depletion studies were available for review.

Residue depletion studies with non-radiolabelled drug

Cattle

Bath (immersion) treatments

A non-GLP/GCP study was conducted to determine the withdrawal period of an immersion bath product containing 40 % ethion and 10 % cypermethrin in cattle (Bringas *et.al.*). The product was administered as per product label (1 L product diluted in 1000 L water to give a 400 ppm solution of ethion).

The animals used (n = 26) had had no contact with ethion prior to commencement of the study. The animals were split into groups based on gender and weight, to ensure each group had an equal number of males and females and that each group had a similar mean weight. After treatment (a single immersion bath), animals were slaughtered at 15, 29, 43, 57, 69 and 92 days post treatment.

At slaughter, samples of liver, kidney lumbar muscle and perirenal fat were taken for analysis. Samples were processed using a QuEChERS clean-up procedure, and analysed using LC-MS/MS.

Table 2. Ethion concentrations in cattle tissues following single immersion bath treatment (Bringas *et.al.*).

Withdrawal time (days)	Amount of ethion found (µg/kg)			
	Muscle	Kidney	Liver	Fat
15 (control – no treatment)	<LOD	<LOD	<LOD	<LOD
	<LOD	<LOD	<LOD	39
15 (treated)	4	<LLOQ	2.8	>ULOQ
	8	<LOD	2.5	>ULOQ
	12	<LLOQ	3.8	>ULOQ
	4	<LOD	2.9	>ULOQ
29	<LOD	5	<LOD	>ULOQ
	<LLOQ	8	<LOD	>ULOQ
	<LOD	9	<LOD	>ULOQ
	<LOD	5	<LOD	>ULOQ
43	<LOD	<LOD	<LOD	12
	<LOD	<LOD	<LOD	82
	<LOD	<LOD	<LOD	12
	<LOD	<LOD	<LOD	28
57	<LOD	<LOD	<LOD	37
	<LOD	<LOD	<LOD	4.5
	<LOD	<LOD	<LLOQ	7.6
	<LLOQ	<LLOQ	<LLOQ	20
69	<LOD	<LOD	<LOD	46
	<LOD	<LOD	<LOD	<LOD
	<LOD	<LOD	<LOD	8
	<LOD	<LOD	<LOD	<LLOQ
92	<LOD	<LOD	<LOD	<LOD
	<LOD	<LOD	<LOD	<LOD
	<LOD	<LOD	<LOD	<LOD
	<LOD	<LOD	<LOD	<LOD
LOD =	1.0	2.0	2.0	2.0
LLOQ =	2.0	4.0	4.0	5.0
ULOQ =	100	100	100	100
Validation range = 5 – 100 µg/kg (Determined using a 6-point calibration curve, in each matrix).				

The Committee noted that ethion residues were most persistent in the fat samples. However, since the results outside the calibration range of the method are unquantifiable, no meaningful depletion curve can be produced. Ethion metabolites were not measured in this study.

Results from another non-GLP/GCP ethion immersion bath study were provided (Anon (2)). Cattle were exposed to ethion via bath treatment containing 40 % ethion and 10 % cypermethrin. The immersion bath was prepared according to label directions (mixing 1 L of ethion/cypermethrin concentrated solution in 1000 L of water), resulting in ethion concentrations of 400 ppm (mg/L). Cattle were treated twice, 9 days apart. The actual ethion dose for each animal could not be determined due to the nature of the dosing regimen

(immersion bath). Four groups of 4 animals (1 male and 3 female) each were slaughtered at 5, 10, 15 and 20 days after the final treatment. Samples of 300 g of muscle (loin), kidney, liver and subcutaneous and perirenal fat were taken from each animal. The analytical method used (multi-residue for OPs) was GC-FPD (flame photometric detector) and was applied to all tissues (fat, liver, muscle and kidney).

Table 3. Ethion concentrations in cattle tissues following 2nd immersion bath treatment (Anon-2).

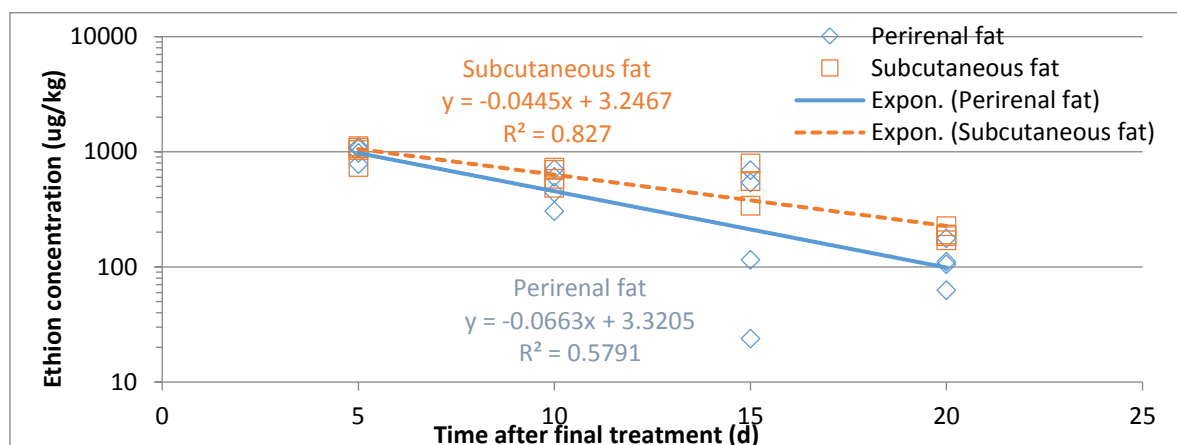
Slaughter time post 2 nd dose (d)	Muscle (µg/kg)	kidney (µg/kg)	Liver (µg/kg)	Perirenal Fat (µg/kg)	Subcutaneous Fat (µg/kg)
5	ND	ND	ND	1107	742
	ND	ND	ND	1072	1090
	ND	ND	ND	979	1039
	<LOQ	ND	ND	782	1125
10	ND	ND	ND	710	697
	<LOQ	<LOQ	ND	306	585
	ND	187	ND	607	489
	ND	<LOQ	ND	444	732
15	ND	ND	ND	695	793
	ND	ND	ND	540	341
	ND	ND	ND	24	No sample
	ND	ND	ND	116	557
20	ND	ND	ND	106	192
	ND	ND	ND	178	186
	ND	ND	ND	111	171
	ND	ND	ND	63	227
LOD* =	6.5	6.5	6.5	6.5	6.5
LOQ* =	19.5	19.5	19.5	19.5	19.5

ND = not detectable (results < LOD).

* See discussion below

The Committee noted that in the study report, the LOD and LOQ for the analytical method used were stated as being 6.5 µg/kg and 19.5 µg/kg, respectively, for all tissue matrices (kidney, liver, fat, muscle). However, in the accompanying method validation study, only data for liver and fat matrices were included, and the LOD was reported to be 3.4 µg/kg for liver and 8.0 µg/kg for fat, and the LOQs were reported as 10.1 µg/kg for liver and 23.9 µg/kg for fat.

Figure 6 shows the depletion profile in the two types of fat over the duration of the study (20 days post final treatment). Residues were more persistent in subcutaneous fat than in peri-reneal fat over the time period studied. As the final sampling group was slaughtered at 20 days, and ethion residues persist for significantly longer duration in fat, the applicability of this data for establishing MRLs is minimal. The data from the other tissues were unable to be analysed statistically, since the results were mostly below the LOD of the analytical method used.

Figure 6. Ethion residue depletion in fat samples following 2nd immersion bath treatment (Anon-2)

Data from a third non-GLP/GCP ethion bath immersion study in cattle were provided (Gérez García *et.al.*, 2017). The products used were immersion bath treatments containing 40 mg/L ethion and 10 mg/L cypermethrin in bath final formulation. Cattle were treated 3 times, with 21 days in between each treatment.

Study animals weighed between 364 and 516 kg and were evenly split between males and females. Animals in the first treatment group were slaughtered at the following timepoints after the final treatment: 13, 34, 70, 90, 105 and 117 days. The animals in a 2nd treatment group were slaughtered 117 days after their final treatment.

After slaughter, samples were taken of muscle (two types, loin and semi-membranous), fat (initially only perirenal fat, but subcutaneous fat was also taken from day 70 onwards), kidney and liver. Each sample weighed around 250 g.

Ethion concentrations in tissues were analysed in a first laboratory (Lab A) using GC-ECD, GC-MS and LC-MS/MS. The reported LOD was 1 µg/kg for all tissues and the LOQs were reported as 5 µg/kg for fat (HPLC) and muscle, 10 µg/kg for fat (GC-MS), liver and kidney. All samples were analysed in triplicate and quantified utilising calibration curves in matrix. A blank was performed for each matrix, as well as at least two residue recoveries for the concurrent validation, which functioned as quality control for each batch. The values above were adjusted by concurrent recovery and lipid content (in the fat samples).

Table 4. Ethion concentrations in cattle tissues following 3rd immersion bath treatment (Lab A results, Gérez García *et.al.*, 2017)

Slaughter time post 3 rd dose (days)	Sex (m/f)	Residues found (µg/kg)				
		Fat		Muscle		Kidney
		Perirenal	Sub-cutaneous	Semi-membranous	Loin	
13	M	1431	NS	NS	32	<10
	F	810	NS	NS	<5	13
	F	1600	NS	NS	23	11
	M	728	NS	NS	<5	<10

34	F	184	70	NS	55	8
	F	797	460	NS	62	6
	M	337	217	NS	47	20
	M	563	392	NS	43	11
70	F	27	32	<LOD	<LOD	7
	F	77	623	<LOD	16	8
	M	272	220	<LOQ	<LOQ	14
	M	29	20	<LOD	<LOQ	8
90	M	13	20	<LOD	<LOD	12
	M	<LOQ	54	<LOQ	<LOQ	<LOQ
	F	<LOQ	72	<LOD	<LOD	<LOQ
	F	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
105	F	<LOQ	<LOQ	<LOD	<LOD	<LOD
	F	<LOQ	<LOQ	<LOD	<LOD	<LOD
	M	<LOQ	<LOQ	<LOD	<LOD	<LOD
	M	12	41	<LOQ	<LOQ	<LOD
LOD		1	1	1	1	1
LOQ		5	5	5	5	10

Notes: All liver results from all timepoints were <LOD (1 µg/kg).

Results from all samples from timepoint 117 days (groups 1 & 2) were <LOD in all tissues (1 µg/kg).

NS = no sample analysed.

In addition, select tissue samples were re-analysed at a second laboratory (Lab B), but only muscle and fat samples from slaughter day 34 onwards. Samples were analysed by LC-MS/MS at the second laboratory, with an LOQ of 1 µg/kg and an LOD of 0.5 µg/kg.

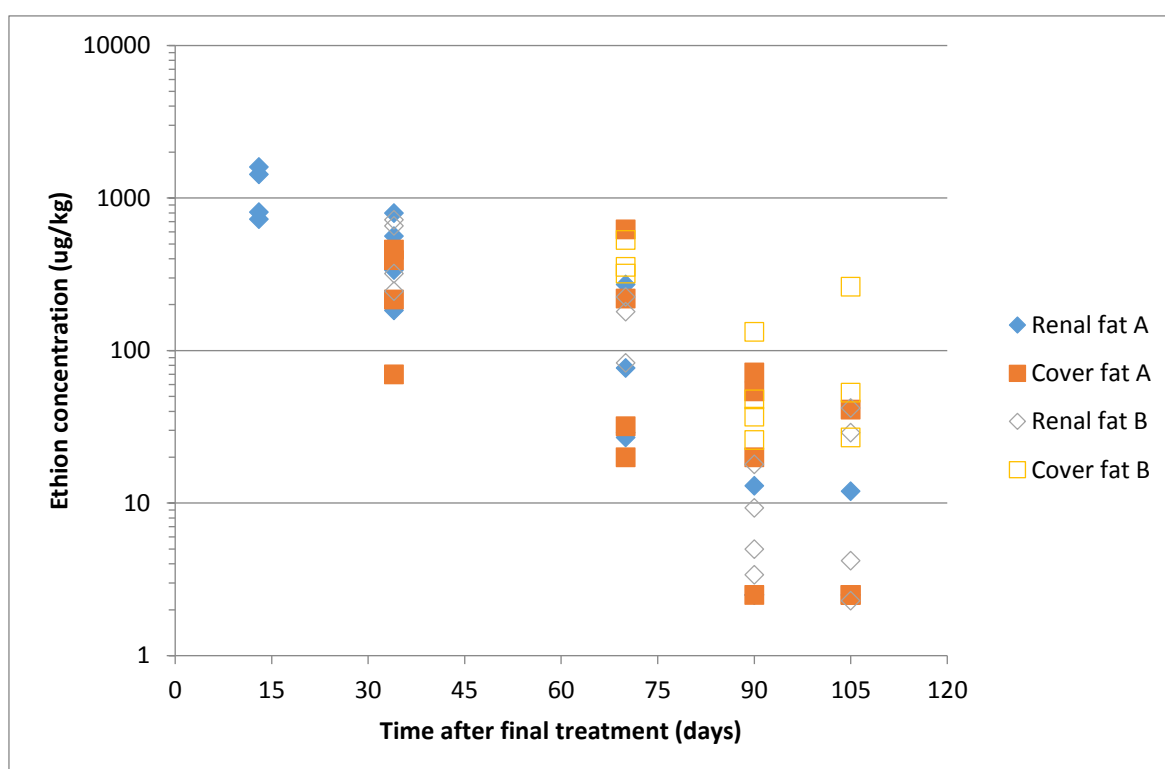
Table 5. Ethion concentrations in select cattle tissues following 3rd immersion bath treatment (Lab B results, Gérez García *et.al.*, 2017)

Slaughter time post 3 rd dose (days)	Sex (m/f)	Residues found (µg/kg)					
		Fat		muscle			
		Perirenal	Sub-cutaneous	Semi-membranous		Loin	
				Lean	15 % fat	Lean	15 % fat
34	F	320	NS	NS	NS	1.1	NS
	F	246	NS	NS	NS	2.0	NS
	M	658	NS	NS	NS	3.6	NS
	M	722	NS	NS	NS	2.5	NS
70	F	83	321	1	NS	1.2	21
	F	180	532	<LOQ	9	1.1	33
	M	225	355	1.1	13	1.3	15
	M	NS	NS	<LOQ	NS	10	NS
90	M	18	37	<LOD	<LOQ	<LOD	1.5
	M	9.3	48	<LOD	2.0	<LOD	1.9

105	F	5.0	133	<LOD	<LOQ	<LOD	<LOQ
	F	3.4	26	<LOD	<LOD	<LOD	<LOD
	F	2.3	27	<LOD	<LOD	<LOD	<LOD
	F	4.2	53	<LOD	<LOD	<LOD	1.2
	M	29	NS	<LOD	NS	<LOD	NS
	M	42	263	<LOD	4.8	<LOD	1.7
LOD		0.5	0.5	0.5	0.5	0.5	0.5
LOQ		1	1	1	1	1	1

Notes: Results from all samples from timepoint 117 days (groups 1 & 2) were <LOD in all tissues (0.5 µg/kg), except one SC fat sample from Group 2 (reported as 3 µg/kg).
NS = no sample analysed.

Figure 7. Ethion residue depletion in fat samples following 3rd immersion bath treatment (Gérez García *et.al.*, 2017)



The Committee noted that the residue concentrations determined in the same tissue sample by the two different laboratories were often highly discrepant. This may be due to differences in analytical methodology (though both assays were purportedly validated), or due to non-homogenous ethion concentrations in different regions of the same tissue sample. However, it is noted that the results were consistent in terms of order of magnitude. It is clear that ethion residues are more persistent in fat than any other tissue sampled, particularly demonstrated by the comparison between lean muscle samples and those containing 15 % fat.

When comparing the results from all three submitted ethion immersion bath studies, the Committee highlighted the following:

- All treatments consisted of almost total immersion of the cattle in the insecticide solution;
- All products used were of the same quantitative composition with respect to the active substances (40 % ethion and 10 % cypermethrin, diluted in water to give 400 ppm solutions of ethion at point of administration);
- Each of these studies used a different treatment protocol. In one study, the animals were treated once, in another study, they were treated twice, with a 9-day interval between treatments, and in the third study, the animals were treated three times, with 21-day intervals between treatments.
- In none of the studies was it possible to determine the exact dose received by each animal.
- In all of the studies, ethion parent molecule was the marker residue (i.e. no metabolites were investigated).
- All studies demonstrated that ethion is most persistent in fat of cattle, and that it persists in cover (subcutaneous) fat for longer than in peri-renal fat.
- Overall, the studies provided were well-conducted and well-reported. All the studies reviewed were designed to calculate a suitable withdrawal period for the products investigated, and were not primarily designed for the derivation of MRLs.

Ear-tag treatments

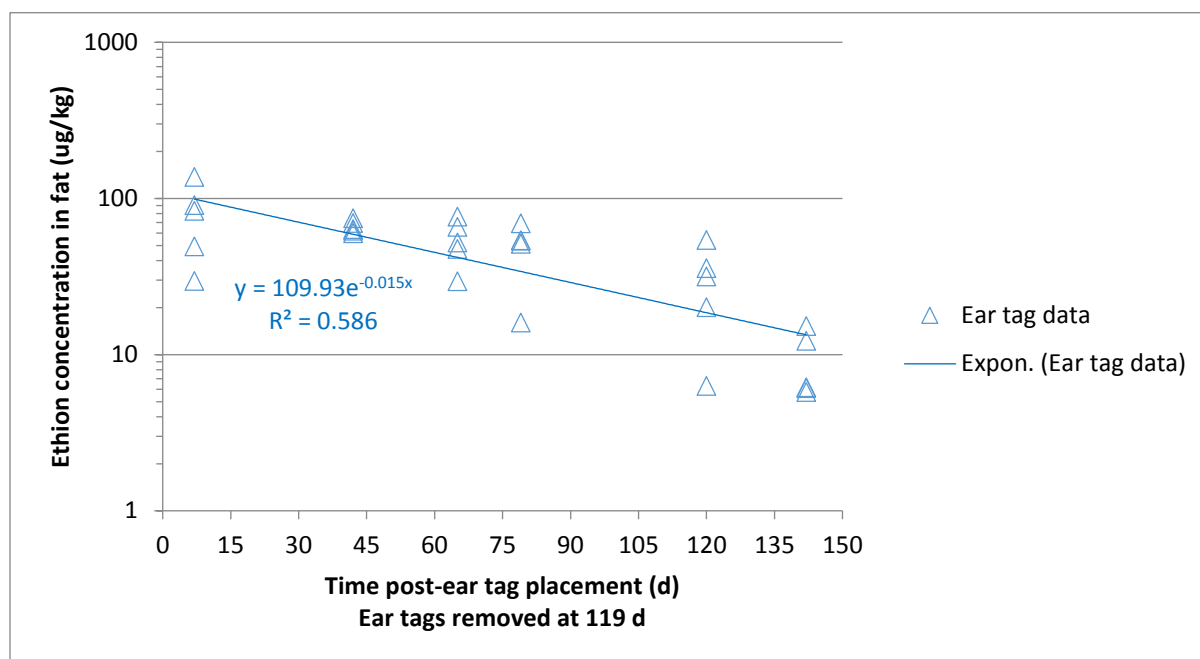
A non-GLP (claimed to be GCP) study in 30 cattle was performed with application of one ear tag (containing 40 g ethion per 100 g ear tag) per animal (Prazeres Gonçalves *et.al.*, 2012). The ear tags remained in place for 119 days. Each group was slaughtered on the following days after application: D +7, D +42, D +65, D +79 (during treatment), D +120 and D +142 (1 and 23 days, respectively, after removal of the ear tags on D +119). Samples of fat, muscle, kidney and liver were taken from each animal for analysis (anatomic location of muscle and fat samples not provided). The samples were processed and stored below -20 °C until analysis via UPLC-MS/MS. Concurrent QC sample data were provided, with the majority of results within -15 to +15 %

No raw data was provided to the Committee.

Table 6. Ethion concentrations in cattle tissues following ear-tag administration (Prazeres Gonçalves *et.al.*, 2012).

Time (days post ear tag application)	Mean residues (n = 2) (µg/kg)			
	Fat	Muscle	Kidney	Liver
7	49.45	<LOQ	<LOQ	<LOD
Tag in ear	137.92	<LOQ	<LOQ	<LOD
	29.67	<LOD	<LOQ	<LOD
	83.73	<LOQ	<LOQ	<LOD
	90.89	<LOQ	<LOQ	<LOD
42	74.78	<LOQ	<LOQ	<LOD
Tag in ear	62.23	<LOD	<LOQ	<LOD
	59.98	<LOQ	<LOQ	<LOD
	63.77	<LOD	<LOQ	<LOD
	69.78	<LOD	<LOQ	<LOD
65	29.59	<LOD	<LOQ	<LOD
Tag in ear	47.72	<LOD	<LOQ	<LOD
	65.97	<LOD	<LOQ	<LOD
	76.86	<LOD	<LOQ	<LOD
	52.27	<LOD	<LOQ	<LOD
79	54.22	<LOD	<LOQ	<LOQ
Tag in ear	69.36	<LOQ	<LOQ	<LOD
	53.95	<LOQ	8.2	<LOQ
	16.12	<LOQ	<LOQ	<LOD
	51.29	6.04	<LOQ	<LOD
119	54.33	14.46	<LOQ	<LOD
(+1 d after tag removed)	31.70	<LOQ	<LOD	<LOD
	20.16	<LOQ	<LOD	<LOD
	35.85	5.53	<LOD	<LOD
	6.32	<LOD	<LOD	<LOD
142	12.28	<LOD	<LOD	<LOD
(+23 d after tag removed)	5.77	<LOD	<LOQ	<LOD
	6.2	<LOD	<LOD	<LOD
	15.24	<LOD	<LOD	<LOD
	6.12	<LOD	<LOQ	<LOD
LOD =	0.82	0.82	0.74	1.01
LOQ =	5.0	5.0	5.0	5.0

Figure 8. Ethion residue depletion in fat samples following ethion ear tag placement (Prazeres Gonçalves *et.al.*, 2012)



The Committee recognised that as with the immersion bath treatments, the residues of ethion persisted longest in fat after ear-tag treatment. Due to the prolonged nature of ethion exposure after ear-tag treatment, the residues persisted longer than those from immersion baths. It should be noted that the depletion of residues was investigated after completion of treatment for the immersion baths, whereas depletion was monitored throughout treatment (and for up to 23 days post-removal) for the ear-tags.

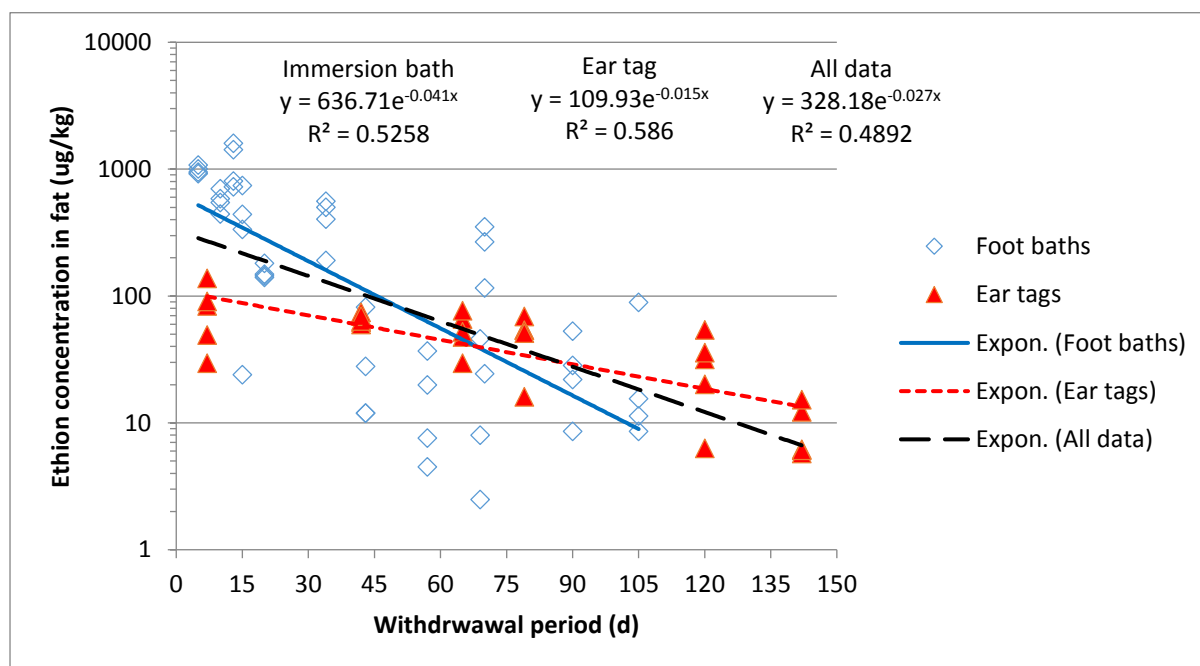
Overall comment on residues depletion studies in cattle tissues:

A brief summary comparison of the various ethion residue depletion studies submitted for evaluation by the Committee are presented in Table 7. Regarding the possibility of combining the data from the various residues depletion studies submitted to the Committee, it was deemed to be inappropriate due to the variety of ethion formulations, dose regimens, sample times, and analytical methodologies used in the different studies. Some rudimentary combination was attempted, however, in order to characterise the differences between treatments (Figure 9). Average results for perirenal/subcutaneous fat, or average of multiple lab results for the same fat sample, are reported.

Table 7. Summary of available ethion residue depletion studies submitted for evaluation by the Committee.

No. of cattle	Ethion dose	Tissues collected	Sampling Times	Assay used	LOD & LOQ (µg/kg)	Issues	Reference
26	400 ppm bath (single administration)	liver, kidney lumbar muscle and perirenal fat	15 – 92 d post dose	LC-MS/MS	1-2, 5	ULOQ only 100 µg/kg – early fat samples above this range. Cannot use to model residue depletion in fat.	Bringas <i>et.al.</i> ,
16	400 ppm bath (2x, 9 d apart)	muscle, kidney, liver and subcutaneous and perirenal fat	5 – 20 d post final dose	GC-FPD	6.5, 19.5	1. LOD/LOQ are different in validation report 2. Sampling duration too short to get true residue depletion curve in fat	Anon(2)
34	400 ppm bath (3x, 21 d apart)	Muscle (2x), kidney, liver, fat (2x)	13 – 105 d post final dose	GC-ECD, GC-MS and LC-MS/MS	1, 5 – 10 (1 st lab) 0.5, 1 (2 nd lab)	1. Products used were not specified 2. Samples analysed at 2 labs with very different results 3. Analytical method validation data for LC-MS/MS were not provided	Gérez García <i>et.al.</i> , 2017
30	40g ethion/100g ear tag	Muscle, kidney, liver, fat, milk	7 – 142 d after ear tag applied. (last 2 samples were 1& 23 d post removal)	UHPLC-MS/MS	0.74 – 1, 5	1. Study not GLP (claims GCP)	Prazeres Gonçalves <i>et.al.</i> , 2012

Figure 9. Combined ethion residue depletion results in fat of cattle following multiple routes of ethion administration.



Statistical evaluation of ethion residue depletion data

The Committee also performed a 95/95 upper tolerance limit (95/95 UTL; upper limit of the one-sided 95 % confidence interval over the 95th percentile of residue concentrations) for all individual ethion residue depletion data sets, in order to assess the usability of such data as part of a future MRL assessment. Results from the most robust data sets (Gérez García *et.al.*, 2017, Prazeres Gonçalves *et.al.*, 2012) are shown in Figures 10 and 11. For the 3x immersion bath 95/95 UTL data in Figure 10, the 95/95 UTLs were derived for both the combined ethion fat residue data (average concentration of both anatomic fat samples from both labs), as well as the most conservative individual data (cover fat concentrations from Lab B).

Figure 10. 95/95 Upper Tolerance Limits of ethion residues in fat after 3x immersion bath (Gérez García *et.al.*, 2017).

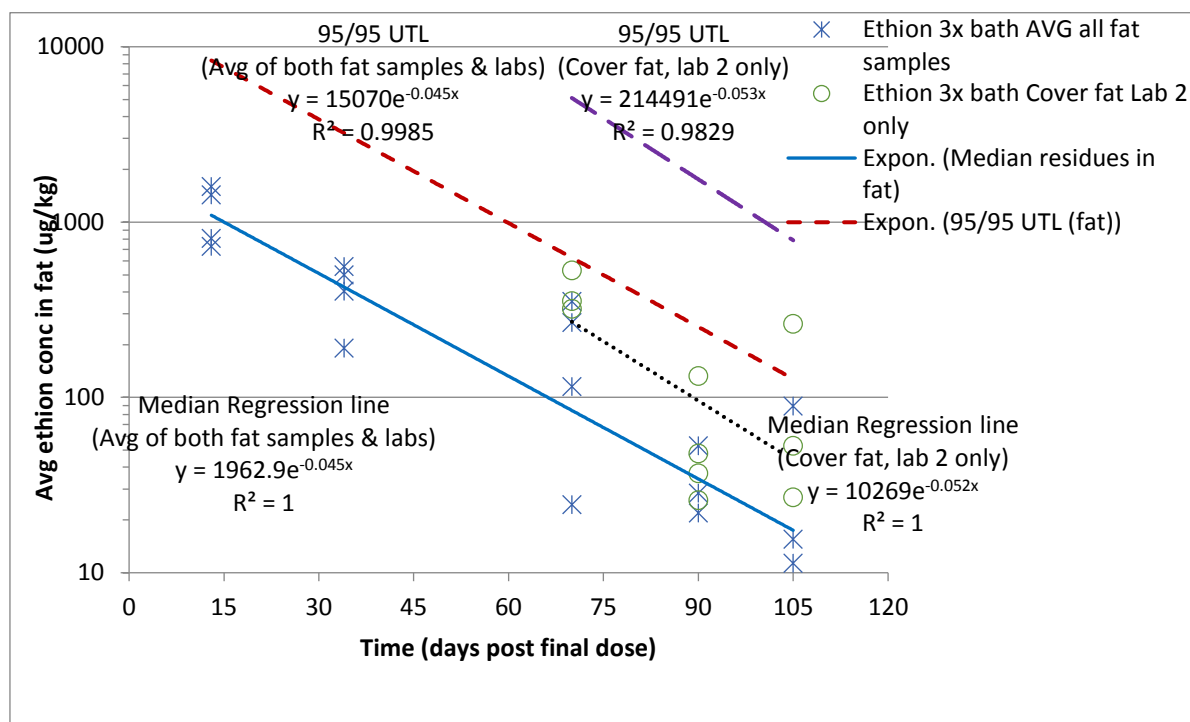
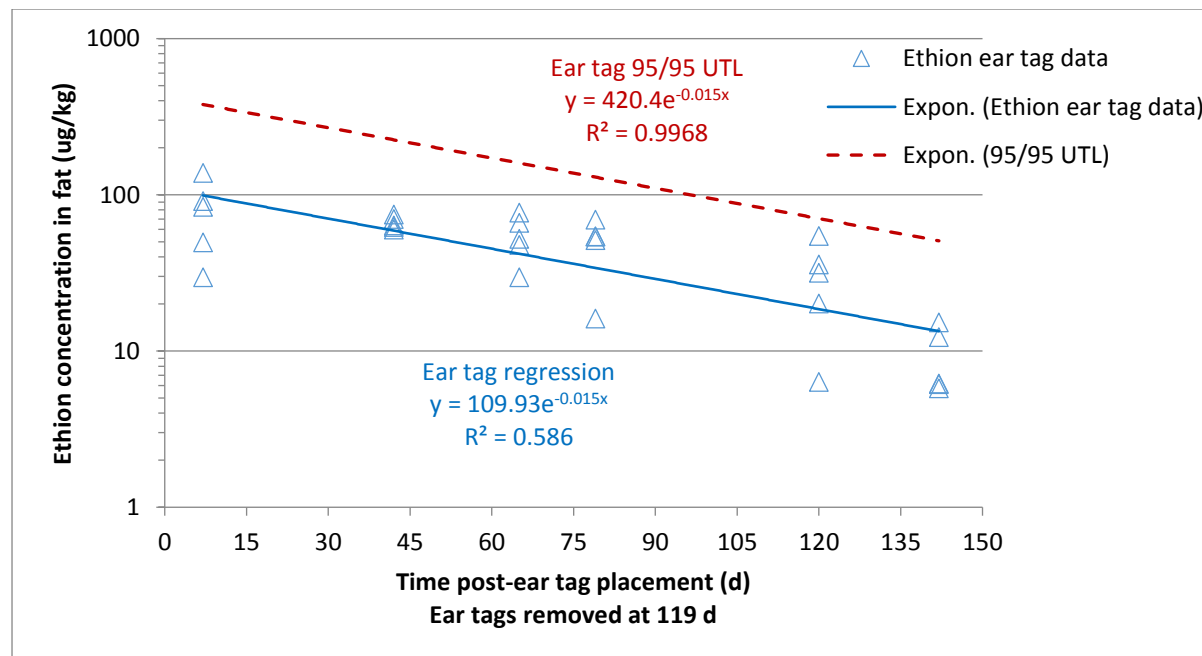


Figure 11. 95/95 Upper Tolerance Limits of ethion residues in fat after ear tag administration (Prazeres Gonçalves *et.al.*, 2012)



Methods of analysis for residues in tissues

Analytical determination of Ethion by GC-FPD, (Bianchini *et.al.*, 2013).

Conducted in accordance with Technique T-CRG-003 Rev.: 07 Analytical determination of organophosphorus pesticides in animal fat and vegetable oil by GC-FPD.

This is a multi-residue analytical technique designed for the detection of many organophosphate insecticides in a single sample of tissue. The tissue is shaken, or the fat is dissolved, in a mixture of hexane-methylene chloride. The analytes are then separated using a gel permeation column where the collected fraction is evaporated to dryness and taken up in a known volume of isooctane. Then it is injected into a gaseous gas chromatograph with flame photometric detector (FPD).

Sample preparation (fat):

Approximately 30 g fat is melted in a microwave for 5 minutes. The molten fat is transferred to a haemolysis tube and stored in a refrigerator until analysis.

Sample preparation (other tissues):

If the matrix is muscle, liver or kidney, the connective tissue and fat is removed as much as possible. The tissue is then cut into small pieces and chopped finely so as to obtain a homogeneous mixture. The samples are then transferred to small plastic bottles (approximately 30 g) and stored in a freezer until analysis.

Extraction:

Two grams of sample is added to 8 ml of hexane:methylene chloride (65:35) solvent and this is vortexed. The eluate is then concentrated by heating and evaporating off the solvent. This concentrate is then added to 1 ml isooctane and injected into the GC-FPD.

Quantification:

The quantification is performed by direct comparison with the injection of a solution containing the standard analytes of interest.

In the case that an unknown sample is positive, a peak with retention time corresponding to the peak of the analyte in the standard (+/- 1 % in seconds) is obtained.

Validation of the method

The method was validated only for the matrices liver and fat, but is applied to all tissues (fat, liver, muscle and kidney).

Table 8: Validation data for GC-FPD, (Bianchini *et.al.*, 2013)

Tissue:	Fat	Liver
Intraday accuracy (recovery %)	23.88 µg/kg: 100 47.76 µg/kg: 92 95.52 µg/kg: 102 143.28 µg/kg: 99 191.04 µg/kg: 105	10.11 µg/kg: 89 20.22 µg/kg: 97
Intraday precision (% CV)	23.88 µg/kg: 9.5 47.76 µg/kg: 9.7 95.52 µg/kg: 9.2 143.28 µg/kg: 7.4 191.04 µg/kg: 5.7	10.11 µg/kg: 8.4 20.22 µg/kg: 9.5
Interday precision	23.88 µg/kg: 7.6 47.76 µg/kg: 7.0 95.52 µg/kg: 6.0 143.28 µg/kg: 8.2 191.04 µg/kg: 3.9	10.11 µg/kg: 8.0 20.22 µg/kg: 6.1
LOQ / LOD (µg/kg)	23.9 / 8.0	10.1 / 3.4
Analytical range	8 – 181 µg/kg	3.4 - 191 µg/kg
Linearity (r²)	0.9993*	
Specificity/selectivity	No interference	
Ruggedness testing	Not affected by the type of matrix used	
Stability:	Not studied	
Freeze-thaw		
Room temperature		
Extract		
Stock solution		

* it has not been specified from which tissue the linearity r² value is from.

Report: Determination of depletion curves of ectoparasitocides in cattle. (Gérez García *et.al.*).

Description of the method

Fat

For both perirenal fat and cover (SC) fat, cryogenic grinding with liquid nitrogen was performed. Extract the analyte from the fat using toluene first and then acetonitrile with shaking. Centrifuge the sample, then cool to -40 °C. Centrifuge again and take the supernatant and add PSA, C18 and anhydrous MgSO₄. Vortex the mixture and then centrifuge again. Take the supernatant and evaporate to dryness for analysis. For GC analysis, redissolve in AcOEt and add the internal standard (bromophos methyl) and inject this solution into the analyser. For LC analysis, redissolve in acetonitrile and put in the freezer overnight. Filter the solution and inject into the LC apparatus.

Kidney

Add homogenised kidney sample to AcOEt and shake in a vortex. Add MgSO₄ and NaCl and shake vigorously. Centrifuge the sample and then cool to -40 °C. Centrifuge again and add d-SPE salts (C18, alumina and MgSO₄). Shake vigorously and remove the supernatant. Evaporate to dryness and add this to a solution of the internal standard in AcOEt, ready for the autosampler.

Muscle

Muscle samples were prepared according to Souza *et.al.*, Development of a straightforward and cheap ethyl acetate based method for the simultaneous determination of pesticides and veterinary drugs residues in bovine liver and muscle. *Chromatographia* (2016) 79:1101–1112, (not supplied).

Liver

No description of the method was given.

Analytical method validation

Validation parameters were evaluated according to the European commission directorate general for health and food safety, ‘Guidance document on analytical quality control and method validation procedures for pesticides residues analysis in food and feed’ (SANTE/11945/2015).

Fat**Accuracy**

Table 9. Accuracy data for GC-ECD and GC-MS (fat) (Gérez García *et.al.*,)

Nominal spiked level (µg/kg) in fat	Recovery (%)	RSD (%)
100	81	2
250	85	3
500	101	4
1000	84	3

Table 10. Accuracy data for LC-MS/MS (fat) (Gérez García *et.al.*,)

Nominal spiked level (µg/kg)	Recovery (%)	RSD (%)
5	62	6
10	56	10
100	63	/

Table 11. Precision (fat) (Gérez García *et.al.*,)

Nominal spiked level (µg/kg)	Repeatability (%)	Reproducibility (%)
100	2	5
250	3	4
500	5	7
1000	3	5

Method linearity and matrix effect (fat)

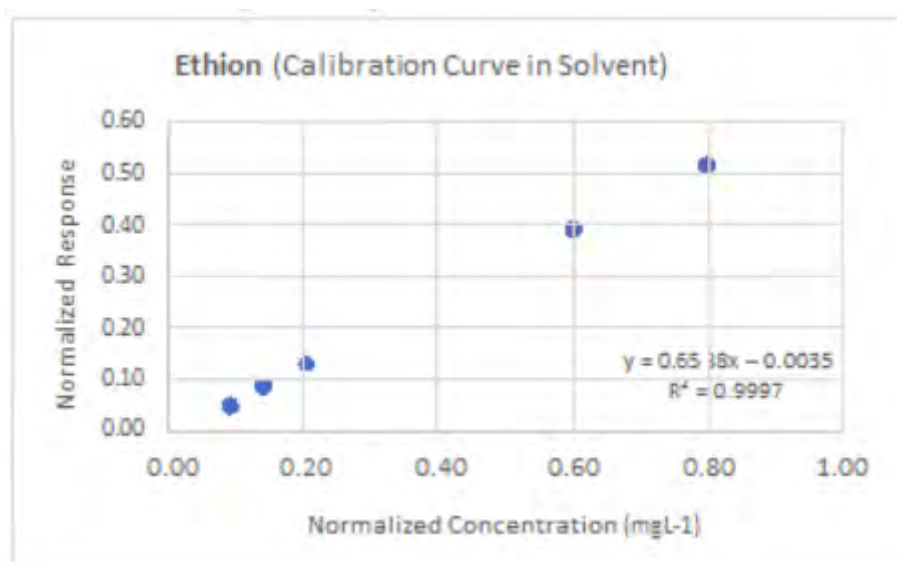
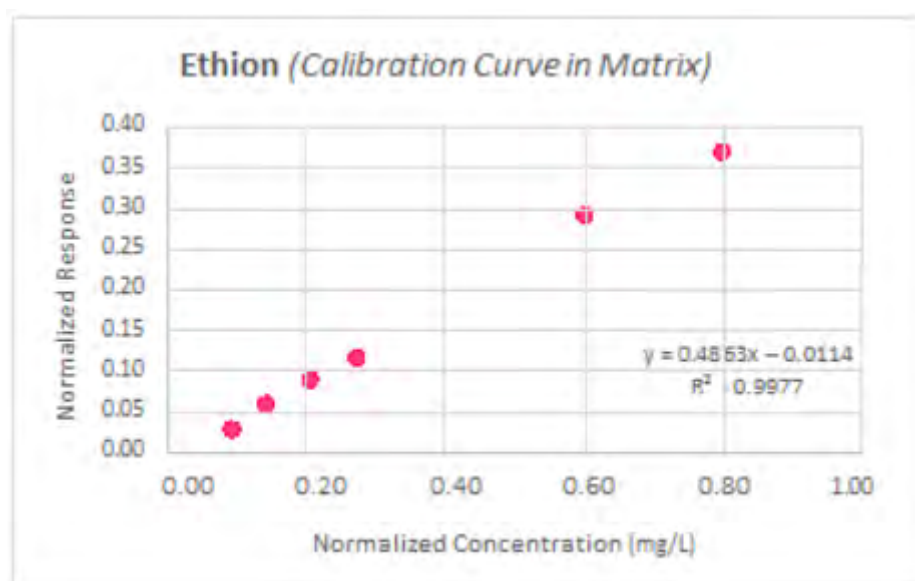
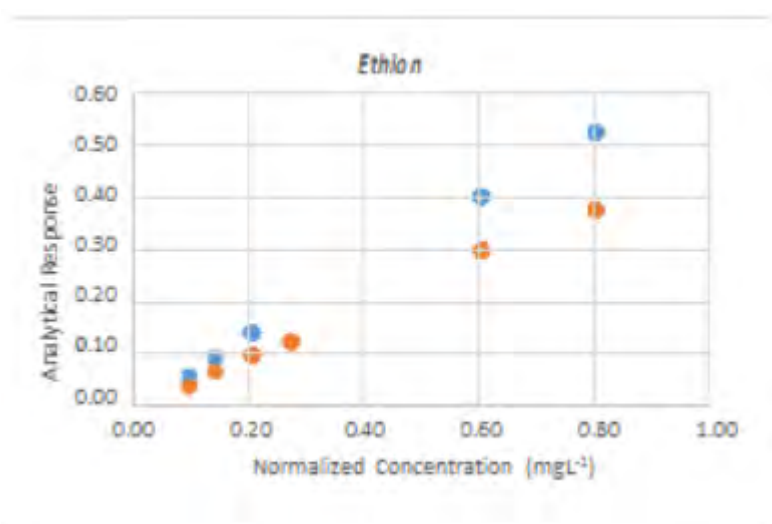
Figure 12. Calibration curve in solvent (GC-ECD) (Gérez García *et.al.*,)**Figure 13.** Calibration curve in matrix (fat) (Gérez García *et.al.*,)

Figure 14. Calibration curves superimposed for visual evaluation (Gérez García *et.al.*,)

A matrix effect in fat of -26 % was obtained using the equation:

$$\text{Matrix effect (\%)} = \frac{\text{slope of the calibration curve in matrix}}{\text{slope of the calibration curve in solvent}} \times 100$$

Kidney

Accuracy

Table 12. Accuracy data for GC-ECD and GC-MS (Gérez García *et.al.*,)

Nominal spiked level (µg/kg)	Recovery (%)	RSD (%)
25	84	5
50	86	4
100	102	8

Table 13. Accuracy data for LC-MS/MS (Gérez García *et.al.*,)

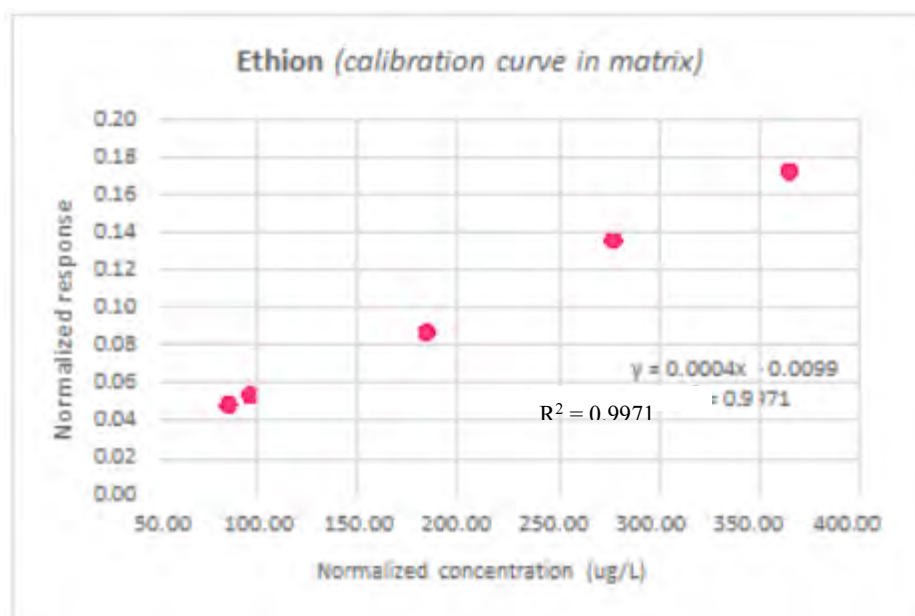
Nominal spiked level (µg/kg)	Recovery (%)	RSD (%)
10	70	6
50	100	5
100	71	11

Table 14. Precision (GC-ECD) (Gérez García *et.al.*,)

Nominal spiked level (µg/kg)	Repeatability (%)	Reproducibility (%)
25	5	7
50	4	9
100	8	12

Table 15. Precision (LC-MS/MS) (Gérez García *et.al.*,)

Nominal spiked level (µg/kg)	Repeatability (%)	Reproducibility (%)
10	5	7
50	4	9
100	8	12

Figure 15. Method linearity (GC-ECD) (Gérez García *et.al.*,)**Figure 16.** Method linearity (LC-MS/MS) (Gérez García *et.al.*,)

A matrix effect in kidney of -51 % was obtained using the equation:

$$\text{Matrix effect (\%)} = \frac{\text{slope of the calibration curve in matrix}}{\text{slope of the calibration curve in solvent}} \times 100$$

No further validation data were given in this report.

LC-MS/MS

The following data were provided in tables untranslated from the original Spanish. No validation report was provided. The LC-MS/MS method was used in the study reported by Bringas *et.al.*, in cattle tissues.

Table 16. Summary of the validation data for the analytical method used in the study to determine residues of ethion in cattle tissues, as reported by Bringas *et.al.*,

Tissue	Muscle	Fat	Liver	Kidney
Intraday accuracy (% recovery)	5 µg/kg: 101	5 µg/kg: 104	5 µg/kg: 108	5 µg/kg: 111
	10 µg/kg: 98	10 µg/kg: 104	10 µg/kg: 106	10 µg/kg: 97
	100 µg/kg: 101	100 µg/kg: 99	100 µg/kg: 92	100 µg/kg: 95
Intraday precision (% CV)	5 µg/kg: 10	5 µg/kg: 4	5 µg/kg: 2	5 µg/kg: 3
	10 µg/kg: 8	10 µg/kg: 1	10 µg/kg: 2	10 µg/kg: 1
	100 µg/kg: 5	100 µg/kg: 9	100 µg/kg: 3	100 µg/kg: 4
Interday accuracy (% recovery)		5 µg/kg: 108.3	5 µg/kg: 108	
		10 µg/kg: 103	10 µg/kg: 105.3	
		100 µg/kg: 95.7	100 µg/kg: 94.3	
Interday precision (% CV)		5 µg/kg: 4.84	5 µg/kg: 1	
		10 µg/kg: 1.09	10 µg/kg: 2.76	
		100 µg/kg: 7.16	100 µg/kg: 2.69	
LOQ / LOD (µg/kg)	2 / 1	5 / 2	4 / 2	4 / 2
Analytical range	5 – 100 µg/kg	5 – 100 µg/kg	5 – 100 µg/kg	5 – 100 µg/kg
Linearity (r²)	0.997	0.9974	0.9948	0.9958
Specificity	No interference	No interference	No interference	No interference
*Stability:				
a) Freeze-thaw		a)	a)	
b) Room temperature (24 h)		b)	b)	
c) Storage (3 °C, 48h in liver and 72h in fat)		c) 10 µg/kg: -3.9 % 100 µg/kg: 6.5 %	c) 10 µg/kg: 2 % 100 µg/kg: 10 %	
d) Long term storage		d)	d)	

* It is stated that stability at room temperature for 24 h has been verified; however, no results have been provided. It has not been specified if the stability at room temperature was tested for the analyte in matrix samples or in the extract after the extraction procedure. In addition, neither freeze/thaw nor long term stability data have been provided.

Validation of the analytical methodology for the determination of Ethion residues in milk and bovine tissues by UPLC/MS/MS (Protocol number: RES-VAL-014/11). (Prazeres Gonçalves *et.al.*, 2012)

Description of the method

This analytical method is based on the coupling of ultra-high pressure liquid chromatography and tandem mass spectrometry (UPLC-MS/MS), and is prepared for the detection and quantification of ethion and fenthion (internal standard) in bovine tissue and milk samples.

Sample preparation:

After vortexing and ultrasound mixing, the samples are centrifuged at 8000 rpm and the phase containing the analyte is separated. This procedure is repeated twice and the extracts are pooled together. This extract is passed through a silica column and the filtrate is then dried under nitrogen atmosphere. The samples are then reconstituted with methanol, centrifuged at 15000 rpm and filtered on a 0.22 µm membrane just before injection into the UPLC system.

Validation of the method

Validation of the analytical methodology for the determination of Ethion residues in milk and bovine tissues by UPLC/MS/MS (Protocol number: RES-VAL-014/11).

Table 17. Summary of UPLC/MS/MS validation data (Prazeres Gonçalves *et.al.*, 2012)

Tissue	Muscle	Fat	Liver	Kidney	Milk
Intraday accuracy (% bias)	5 µg/kg: 105	5 µg/kg: 96.54	5 µg/kg: 99.22	5 µg/kg: 99.2	5 µg/kg: 103.34
	10 µg/kg: 95.88	10 µg/kg: 107.41	10 µg/kg: 100.21	10 µg/kg: 100.33	10 µg/kg: 93.17
	70 µg/kg: 99.55	70 µg/kg: 94	70 µg/kg: 103.98	70 µg/kg: 103.35	70 µg/kg: 95.84
	100 µg/kg: 100.30	100 µg/kg: 103.7	100 µg/kg: 111.01	100 µg/kg: 112.29	100 µg/kg: 107.65
	250 µg/kg: 105.23	250 µg/kg: 101.74	250 µg/kg: 98	250 µg/kg: 92.77	250 µg/kg: 98.6
	500 µg/kg: 97.03	500 µg/kg: 96.61	500 µg/kg: 87.58	500 µg/kg: 92.06	500 µg/kg: 101.39
Intraday precision (% CV)	10 µg/kg: ≤ 6.36	5 µg/kg: 5.43	5 µg/kg: 6.45	5 µg/kg: 4.72	5 µg/kg: 1.47
	100 µg/kg: ≤ 2.35	10 µg/kg: 5.79	10 µg/kg: 5.40	10 µg/kg: 2.25	10 µg/kg: 2.33
	250 µg/kg: ≤ 7.15	70 µg/kg: 6.91	70 µg/kg: 2.77	70 µg/kg: 4.96	70 µg/kg: 2.02
		100 µg/kg: 2.29	100 µg/kg: 3.45	100 µg/kg: 1.32	100 µg/kg: 1.27
		250 µg/kg: 1.97	250 µg/kg: 3.39	250 µg/kg: 1.26	250 µg/kg: 3.13
		500 µg/kg: 4.05	500 µg/kg: 1.56	500 µg/kg: 2.51	500 µg/kg: 2.52
Interday precision	10 µg/kg: ≤ 3.88				
	100 µg/kg: ≤ 3.85				
	250 µg/kg: ≤ 1.86				
LOQ / LOD (µg/kg)	5 / 0.82	5 / 0.82	5 / 1.01	5 / 0.74	5 / 0.24 (µg/l)
Analytical range (µg/kg)	5 – 500	5 – 500	5 – 500	5 – 500	5 – 500 (µg/l)
Linearity (r²)	0.9965	0.9936	0.9900	0.9914	0.9958
Specificity	< 1.5 %	No interference	No interference	< 0.52 %	< 0.25 %
Matrix effect	Significant	Significant	Significant	Significant	Significant
Ruggedness testing	3.66 %				
Stability (%):					
a) Freeze-thaw	a) 10 µg/kg: 4.19	a) 10 µg/kg: -12.68	a) 10 µg/kg: -0.51	a) 10 µg/kg: 1.06	a) 10 µg/kg: 4.89
	250 µg/kg: -0.91	250 µg/kg: -12.59	250 µg/kg: -1.22	250 µg/kg: -4.22	250 µg/kg: 3.79
b) Room temperature	b) 10 µg/kg: -1.73	b) 10 µg/kg: -14.26	b) 10 µg/kg: 1.54	b) 10 µg/kg: -10.11	b) 10 µg/kg: 6.73
	250 µg/kg: -0.91	250 µg/kg: -12.76	250 µg/kg: 4.61	250 µg/kg: -1.9	250 µg/kg: 1.43

c) Extract	c) 10 µg/kg: 0.68	c) 10 µg/kg: -0.8	c) 10 µg/kg: -2.17	c) 10 µg/kg: -11.74	c) 10 µg/kg: 0.87
	250 µg/kg: -6.69	250 µg/kg: -1.15	250 µg/kg: 10.38	250 µg/kg: -6.86	250 µg/kg: -1.65
d) Long term storage	d) 10 µg/kg: -6.06	d) 10 µg/kg: 4.75	d) 10 µg/kg: -2.47	d) 10 µg/kg: 5.52	d) 10 µg/kg: 2.25
	250 µg/kg: -4.91	250 µg/kg: -1.27	250 µg/kg: 0.47	250 µg/kg: -5.52	250 µg/kg: 1.38

The validation study was performed by LABTEC (Sao Paulo, Brazil). The final report is dated 13/09/2011. Declaration of conformity with GLP has been provided by the responsible person at the quality assurance team of LABTEC.

A detailed description has been given on how the mobile phase, extraction solution and stock solutions of ethion and fenthion are prepared for the validation study. Sample preparation is as described above.

Linearity, specificity/selectivity, matrix effect, limit of detection, limit of quantification, accuracy and stability were studied in order to validate the analytical method in bovine tissues (muscle, kidney, liver and fat) and milk. Precision and robustness of the method was also analysed in muscle tissues.

Linearity

Linearity of response was studied over the concentration range of 5, 10, 70, 100, 250 and 500 µg/kg (or µg/l for milk), with 7 sample replicates for each concentration. Linearity was confirmed for the method response in solvent, blank matrix and blank matrix extract, spiked with calibration standards. Correlation coefficient (r) was in all cases above 0.98 and residuals errors deviated less than 20 % from the nominal concentration.

Specificity

Specificity were confirmed by the absence of relevant chromatographic peaks in blank matrix samples, as compared to the peak areas of ethion and internal standard fenthion in spiked matrix samples for all the studied tissues.

Matrix effect

The possible interferences on the analysis caused by matrix components or sample extraction procedure were studied comparing the data obtained from the linearity test of calibration curves in blank spiked extracts and in standard solution. Statistical analysis (t-student) revealed significant differences in muscle, fat, kidney, liver and milk, meaning that these matrices have an effect on the precision of the method. Therefore, ethion residue concentrations must be quantified using the matrix calibration curve.

The Limit of Detection

The LOD was calculated by multiplying the standard deviation of the 7 replicates at the lowest concentration level (5 µg/kg) by the t-student t value with n = 7 and confidence level of 99 %. The calculated LODs for different tissues were:

- Muscle: 0.82 µg/kg
- Fat: 0.82 µg/kg

- Kidney: 0.74 µg/kg
- Liver: 1.01 µg/kg
- Milk: 0.24 µg/l

Limit of Quantification

The LOQ was set at 5 µg/kg for all tissues (5 µg/l for milk), corresponding to the lowest level at which accuracy and precision were demonstrated.

Precision (only for muscle tissue)

Repeatability and reproducibility were studied at three different concentrations (10, 100 and 250 µg/kg). For repeatability, six replicates of each concentration were analysed on three different days. Coefficient of variation (CV %) was lower than 2/3 of the theoretical value calculated with the Horwitz equation, except for 250 µg/kg samples of muscle on the second day that presented a slightly higher CV.

For reproducibility, the same number of samples were analysed by a different operator. Mean CV % did not exceed the limit value of 20 % for concentration levels between 10 and 100 µg/kg, or 15 % for concentration levels between 100 and 1 000 µg/kg.

Accuracy

Accuracy was studied by the means of recovery of 7 replicates of sampled fortified at 5, 10, 70, 100, 250 and 500 µg/kg (or µg/l for milk). All values reported and the mean values for each concentration level were within the accepted range for accuracy, irrespective of the type of matrix.

Robustness (only for muscle tissue)

Robustness of the analytical method was confirmed with the Youden test. Seven different parameters were slightly modified (methanol concentration, extraction volume, ultrasound time, vortex mixing time, temperature in the centrifuge, speed of the centrifuge and time in the centrifuge) over 8 analytical runs at 100 µg/kg with different combinations on each run. The mean concentration level obtained on the robustness analysis was similar to that obtained on repeatability studies, thus confirming that the method is robust.

Stability

Stability of the analyte was studied in spiked samples:

- after three consecutive freeze/thaw cycles
- after 24 h at -20 °C and a subsequent 4 h at room temperature
- after 25, 67, 46, 115 and 63 h for muscle, fat, kidney, liver and milk, respectively, at 15 °C on the auto-injector
- after 218, 262, 273 and 241 days for muscle, fat, kidney and milk, respectively, stored at -20 °C

Variation was less than 15 % in all cases when compared to freshly extracted samples.

Comment:

The analytical method used for the quantification of ethion in bovine tissues and milk has been adequately validated in terms of linearity of the response, specificity, precision, accuracy, robustness and stability of the analyte (after freeze/thaw, after short and long term storage, and after extraction process on the auto-injector). LOD was set at 0.82, 0.82, 0.74, 1.01 and 0.24 µg/kg for muscle, fat, kidney, liver and milk, respectively. LOQ was set at 5 µg/kg for all matrices. A matrix effect was observed in all matrices; therefore, quantification of the analyte was done using the matrix calibration curves.

However, calculations for the stock solution preparation are not clear and there are some missing data from long term stability in liver.

Comprehensive Literature search

As part of the residue evaluation of ethion, the Committee performed a comprehensive literature search in April 2017 to identify any information relevant for its assessment. The following online databases were searched: Pubmed, B-ON, Springer Nature, Science Direct and Web of Science.

The following inclusion criteria were applied for determining study relevance:

- Any article regarding ethion concentrations in plasma of cattle or other ruminants;
- Any article regarding ethion concentrations in edible tissues of cattle or other ruminants;
- Any article regarding ethion residue determination methods for cattle plasma/tissue;
- Any article regarding ethion metabolism / metabolites in cattle
- Any article regarding bioavailability of ethion residues in animals
- Articles in all languages were included;

To determine the relevance of the published studies identified in the literature search, the following criteria were used when deciding to exclude studies from the assessment:

- Any article focusing on ethion efficacy against target parasites
- Any article focusing on parasite resistance to ethion
- Any article focusing on ethion use in food animal species other than ruminants;
- Any article focusing on kinetics/residues of organophosphates other than ethion (and not including ethion for comparison)
- Any article focusing on pharmacokinetics or pharmacodynamics of ethion in parasite species

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.

The Committee noted that the majority of relevant papers for the ethion MRL evaluation in cattle concerned specific analytical methodologies, usually for use in national or regional surveillance of residues of pesticides in foods, and usually multi-residue methods. There was some potentially useful information on the stability of ethion in various matrices. There were no papers evaluating the pharmacokinetics or residues depletion of ethion in cattle.

A summary of the relevant recent publications follows:

Analytical methods (meat):

Determination of organophosphorus pesticides in bovine tissue by an on-line coupled matrix solid-phase dispersion–solid phase extraction–high performance liquid chromatography with diode array detection method. Tania M. Gutiérrez Valencia, Martha P. García de Llasera. *Journal of Chromatography A*. 2011. 1218:6869– 6877.

Summary: A miniaturised method based on matrix solid-phase dispersion coupled to solid phase extraction and high performance liquid chromatography with diode array detection (MSPD-SPE-HPLC/DAD) was developed for the trace simultaneous determination of organophosphorus pesticides (OPPs) in bovine tissue. The methodology was validated with liver samples. The LOD was 200 µg/kg, and the limit of quantification was 900 µg/kg.

Committee Comment: This is a well-validated multi-residue method for use in residues surveillance. However, the LOD and LOQ for ethion are not especially sensitive, and as such may not be able to detect or quantify ethion residues at concentrations suitable for MRL assessment. Additionally, it is likely that the target tissue for detection of ethion residues in cattle will be fat, and this method was only validated in liver, with confirmation of method ruggedness in muscle and lung tissues. Finally, the assay was designed for detection of only parent ethion (among other organophosphates), and not any ethion metabolites.

Analytical methods (milk):

1. Pesticide Residues in Bovine Milk in Punjab, India: Spatial Variation and Risk Assessment to Human Health. Bedi *et.al.*, *Archives of Environmental Contamination and Toxicology*, 2015. 69:230-240.

Committee Comment: This paper describes a study of pesticide residues, one of which was ethion, in bovine milk in India (specifically the Punjab region), and their impact on human health through the consumption of milk. The study focused on the pesticide extraction and quantification in only one matrix (milk), and did not evaluate the presence of ethion (or its metabolites) in bovine tissues.

2. Development of a headspace solid-phase microextraction/gas chromatography–mass spectrometry method for determination of organophosphorus pesticide residues in cow milk. Rodrigues *et.al.*, *Microchemical Journal*, 2011. 98:56-61.

Summary: A method based on solid-phase micro-extraction in mode headspace (HS-SPME) coupled to gas chromatography–mass spectrometry (GC–MS) was developed and optimised

through multivariate factorial design to determine residues of organophosphorus pesticides in cow's milk. The evaluated pesticides included ethion. To evaluate residues of these pesticides in milk, cows were exposed to the pesticides of interest and milk was collected after 24 h. The developed method was able to detect trace amounts of these pesticides in the collected milk samples. Ethion could be detected at concentrations below its LOQ (6.5 µg/L) up to 72 h after the spraying of cows.

Committee Comment: This paper demonstrates an analytical technique that could be used to determine ethion residues in milk. The LOD and LOQ (2.2 & 6.5 µg/L, respectively) would be sufficiently sensitive for regulatory purposes. However, it only evaluated organophosphate residues in milk (no bovine tissues), and was not designed for detection of ethion metabolites.

3. Combination of solid-phase extraction with dispersive liquid–liquid microextraction followed by GC–MS for determination of pesticide residues from water, milk, honey and fruit juice. Shamsipur *et.al.*, Food Chemistry, 2016. 204:289-297.

Summary: A pre-concentration method for the extraction and determination of traces of multi-residue pesticides (including ethion) was developed using solid-phase extraction (SPE) coupled with dispersive liquid–liquid microextraction and GC–MS, resulting in a very sensitive method for a variety of liquid matrices.

Committee Comment: Another analytical methodology paper that evaluated organophosphate residues in milk (and other liquids), but not bovine tissues.

4. Selective, solid-matrix dispersion extraction of organophosphate pesticide residues from milk. Di Muccio *et.al.*, Journal of Chromatography A, 1996. 754:497-506.

Summary: Description of a single-step, selective extraction and clean-up of organophosphate (OP) pesticide residues from milk. Recovery experiments were carried out on homogenised commercial milk (3.6 % fat content), spiked with 24 OP pesticides, including ethion.

Committee Comment: Another analytical methodology paper that evaluated organophosphate residues in spiked milk samples, but not bovine tissues.

Stability of ethion in various matrices:

Development and certification of reference materials for residues of organochlorine and organophosphorus pesticides in beef fat ACSL CRM 1 and 2. Armishaw *et.al.*, Fresenius Journal of Analytical Chemistry. 1998. 360:630 - 639.

Summary: Beef fat samples were prepared and tested for three organophosphorus pesticides. Beef fat was spiked with pesticide solutions prepared from certified reference materials (e.g., ethion, 99 %). A sample was prepared containing close to 0.8 mg/kg of each of the organophosphorus pesticides diazinon, chlorpyrifos and ethion. No instability in any of these compounds was detected over a twelve month period.

Comment: This paper is relevant because it comments on the stability of ethion in beef fat; however, the beef fat used in the preparation of the candidate reference materials was first stabilised with 0.02 % w/w butylhydroxyanisole (BHA) to assist with long term storage. Furthermore, the stability results are from beef fat samples spiked with ethion, and not

endogenous residues. No information regarding ethion residue depletion is present in the study. Ethion metabolites were not assessed.

The following published studies were identified in the literature search, but provided no information relevant to ethion residue assessment in bovine tissues:

Table 18. Unused papers from the literature search

Paper	Reason for exclusion
¹⁴ C-Ethion residues in soybean seeds: metabolic pathway, effect of processing, bioavailability, toxicity and protective action of artichoke leaf powder towards rats. Abdel-Gawad, H, <i>et.al.</i> , 2013. Toxicological & Environmental Chemistry, 95:2:288-303.	Not relevant for the determination of residues in cattle matrices.
Residues of ¹⁴ C-Ethion Along the Extraction and Refining Process of Maize Oil, and the Bioavailability of Bound Residues in the Cake for Experimental Animals. H. Abdel-Gawad <i>et.al.</i> , (2013). Environmental Contamination and Toxicology, 2013, 91:240–245.	Not relevant for the determination of residues in cattle matrices.
In vitro evaluation of acaricides efficiency to bovine's ticks of Rio Grande do Sul State, Brazil. Camillo <i>et.al.</i> , Ciência Rural Santa Maria, 2009. 39:2:490-495	Assessment of ethion efficacy
Calculation of Pesticide Degradation in Decaying Cotton Gin Trash. Angus N. Crossan & Ivan R. Kennedy. The Bulletin of Environmental Contamination and Toxicology. 2008. 81:355–359.	Not relevant for the determination of residues in cattle matrices.
Effects of different spray formulations on the reproductive parameters of engorged Rhipicephalus (Boophilus) microplus females detached from experimentally infested cattle. Cruz <i>et.al.</i> , Preventative Veterinary Medicine. 2015. 122:70-75.	Assessment of efficacy
LC-MS/MS Determination of Organophosphorus Pesticide Residues in Coconut Water. Deme <i>et.al.</i> , Food Anal. Methods. 2013. 6:1162–1169.	Not relevant for the determination of residues in cattle matrices.
Distribution, fate and histopathological effects of ethion insecticide on selected organs of the crayfish, Procambarus clarkia. Desouky <i>et.al.</i> , Food and Chemical Toxicology. 2013. 52:42-52.	Not relevant for the determination of residues in cattle matrices.
Extraction of organophosphorus pesticides by carbon-coated Fe ₃ O ₄ nanoparticles through response surface experimental design. Maddah <i>et.al.</i> , Journal of Separation Science. 2016. 39:256–263.	Analytical method development – too technical for this review.

Monitoring of organochlorine and organophosphorus pesticide residues in water during different seasons of Tighra reservoir Gwalior, Madhya Pradesh, India. Mamta <i>et.al.</i> , Environmental Monitoring and Assessment. 2015. 187:684	Not relevant for the determination of residues in cattle matrices.
A single method for detecting 11 organophosphate pesticides in human plasma and breastmilk using GC-FPD. Naksen <i>et.al.</i> , Journal of Chromatography B. 2016. 1025:92-104.	Not relevant for the determination of residues in cattle matrices.
Rapid determination of residues of pesticides in honey by μ GC-ECD and GC-MS/MS: Method validation and estimation of measurement uncertainty according to document No. SANCO/12571/2013. Paoloni <i>et.al.</i> , Journal of Environmental Science and Health, Part B. 2017. 51:3:133-142.	Not relevant for the determination of residues in cattle matrices.
Determination of coumaphos, chlorpyrifos and ethion residues in propolis tinctures by matrix solid-phase dispersion and gas chromatography coupled to flame photometric and mass spectrometric detection. Pérez-Parada <i>et.al.</i> , Journal of Chromatography A. 2011. 1218:5852-5857.	Not relevant for the determination of residues in cattle matrices.
Validation of a matrix solid phase dispersion (MSPD) technique for determination of pesticides in lyophilized eggs of the chicken <i>Gallus gallus domesticus</i> . Reis Souza <i>et.al.</i> , Microchemical Journal. 2013. 110:395-401.	Not relevant for the determination of residues in cattle matrices.
Determination of the Residue Levels of Some Commonly Used Organophosphorus Pesticides in Breast Milk. Şahin <i>et.al.</i> , Ankara Medical Journal. 2017. 1:9-20.	Not relevant for the determination of residues in cattle matrices.
Determination of organophosphorus pesticide residues in vegetables using solid phase micro-extraction coupled with gas chromatography-flame photometric detector. Sapahin <i>et.al.</i> , Arabian Journal of Chemistry. 2014. In press.	Not relevant for the determination of residues in cattle matrices.
Effect of acaricides on the activity of a <i>Boophilus microplus</i> glutathione S-transferase. Silva Vaz <i>et.al.</i> , Veterinary Parasitology. 2004. 119:237-245.	Assessment of ethion efficacy
Rapid method for the determination of some organophosphorus insecticides in a small amount of serum in emergency and occupational toxicology cases. Singh & Dogra, Indian Journal of Occupational and Environmental Medicine. 2009. 13:2:84-87.	Not relevant for the determination of residues in cattle matrices.
Levels of select organophosphates in human colostrum and mature milk samples in rural region of Faizabad district, Uttar Pradesh, India. Srivastava <i>et.al.</i> , Human and Experimental Toxicology. 2011. 30:10:1458-1463.	Not relevant for the determination of residues in cattle matrices.

Analysis of organophosphorus pesticides in whole blood by GC-MS- μ ECD with forensic purposes. Valente <i>et.al.</i> , Journal of Forensic and Legal Medicine. 2015. 33:28-34.	Not relevant for the determination of residues in cattle matrices.
Effects of co-existed proteins on measurement of pesticide residues in blood by gas chromatography–mass spectrometry. Yue <i>et.al.</i> , Journal of Chromatography B. 2010. 878:3089-3094.	Not relevant for the determination of residues in cattle matrices.
Short Communication: Evaluation of insecticide ear tags containing ethion for control of pyrethroid resistant <i>Haematobia irritans</i> (L.) on dairy cattle. Anziani <i>et.al.</i> , Veterinary Parasitology. 2000. 91:147-151	Assessment of ethion efficacy
Silica nanoparticle based techniques for extraction, detection, and degradation of pesticides. Bapat <i>et.al.</i> , Advances in Colloid and Interface Science. 2016. 237:1-14.	Analytical method development for pesticide extraction from environmental samples.
Matrix solid phase dispersion (MSPD), Steven A. Barker The Journal of Biochemical and Biophysical Methods. 2007. 70:151–162.	Analytical method development.
Matrix solid-phase dispersion as a valuable tool for extracting contaminants from foodstuffs. Sara Bogialli & Antonio Di Corcia. The Journal of Biochemical and Biophysical Methods. 2007. 70:163–179.	Analytical method development.
Use of gas-liquid chromatography with electron-capture and thermionic-sensitive detection for the quantitation and identification of pesticide residues. Sicbaldi <i>et.al.</i> , Journal of Chromatography A. 1997. 765:13-22.	Analytical method development.
Prioritization of pesticides based on daily dietary exposure potential as determined from the SHEDS model. Melnyk <i>et.al.</i> , Food and Chemical Toxicology. 2016. 96:167-173.	Exposure assessment.
Long-term stability of pure standards and stock standard solutions for the determination of pesticide residues using gas chromatography, Avramides, EJ., Journal of Chromatography A. 2005. 1080:166 – 176.	Stability study for ethion stock solutions; analytical method was described but not validated.

Appraisal

Ethion is a small, lipid-soluble compound that can be absorbed by passive diffusion through the lungs, gastrointestinal tract, and skin. Absorption appears to be rapid by the oral route, but is slower via the dermal route due to its deposition in the epidermis and subcutaneous fat layer. Ethion is desulfurated by cytochrome P450 enzymes in the liver to its recognised active form, ethion monoxon, which causes acute toxicity due to its potent inhibition of neural acetylcholinesterase. Ethion and its monoxon metabolite are further metabolised by the action of esterases in the blood and liver, producing a variety of metabolites that have not been fully characterised. Elimination is mainly through excretion of water-soluble but uncharacterised metabolites in the urine.

No pharmacokinetic data were available for the target species, cattle.

Toxicokinetic parameters and cumulative excretion were studied in goats after intravenous, oral and dermal administration of unlabelled and ^{14}C -ethion (Mosha *et.al.*, 1991).

- After IV injection of 2 mg/kg bw, the elimination half-life ($t_{1/2}$) was 2 h, total body clearance (Cl_t) was 3.2 L/kg/h and the volume of distribution (Vd_{ss}) was 9.4 L/kg. Plasma concentrations of ^{14}C -ethion (ethion + metabolites) were much higher and more persistent than those of parent ethion, likely indicating the presence of significant quantities of ethion metabolites in plasma. Cumulative excretion of ^{14}C -ethion was 78 % of the dose with 66 % in urine, 8 % in faeces and 4 % in milk over 14 days.
- Oral administration of 10 mg/kg bw resulted in low plasma concentrations of parent ethion and an oral bioavailability of less than 5 %. Cumulative excretion over 14 days was 80 % of the dose with 64 % in urine, 14 % in faeces and 1.7 % in milk.
- Dermal application of 100 mg/kg bw demonstrated a prolonged absorption half-life (85 h) and a bioavailability of 20 %. Only 0.05 % of the dose was excreted unchanged in milk.

It was concluded that (1) orally administered ethion is extensively metabolised in the gastrointestinal tract, (2) dermal application results in extended systemic absorption due to deposition in the dermal fat layer and (3) systemically absorbed parent ethion is rapidly metabolised. The metabolites were not identified in this study.

Residue data

No radiolabelled residue depletion studies in cattle, or any other species, were available for review.

There were no data available for pour-on or spray-on products.

There were four studies investigating the depletion of ethion residues in cattle tissues after dermal administration (via immersion bath or ear tag) provided by the requesting member states; these were reviewed by the Committee (see Table 7). None of these studies investigated the depletion of any of the metabolites and used parent ethion as the marker residue.

The Committee did not have access to the raw data for any of these studies, relying instead on either English translations of the study reports, which were originally in either Spanish or Portuguese, or on evaluation reports from the member states in which the products are authorised.

One study (Prazeres Gonçalves, 2012) was provided that investigated residues during and after treatment with an ear-tag product (40 g ethion per tag). Thirty cattle, divided into 6 groups of 5 animals each, were treated on day zero (D0), with one ear tag applied per animal and remaining in place for 120 days. The groups were slaughtered on days 7, 42, 65 and 79 (whilst the tags were in place), and on days 120 and 142 (1 and 23 days, respectively, after removal of the ear tags on day 119). Samples of fat, muscle, kidney and liver were taken for analysis by ultra-high performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS). Residues were highest in fat samples, although it was not recorded from which anatomic location the fat samples had been taken (i.e., subcutaneous or perirenal). See Table 6 for complete results.

Three residue depletion studies that used immersion bath treatments were provided (Bringas *et.al.*, (undated), Anon (2, undated), Gérez García *et.al.*, 2017). These treatments consisted of almost total immersion of the cattle in the insecticide solution. All the products used were of the same quantitative composition with respect to the active substances (40 % ethion and 10 % cypermethrin concentrates, mixed in water to give a 400 ppm concentration of ethion at point of administration). Each of these studies used a different treatment protocol. In one study, the animals were treated once, in another study, they were treated twice, with a 9-day interval between treatments, and in the third study, the animals were treated three times, with 21-day intervals between treatments. In none of the studies was it possible to determine the exact dose received by each animal.

In the first study (Bringas *et.al.*), the animals were treated once. Groups of four animals were slaughtered on days 15, 29, 43, 57, 69 and 92 days post treatment. There were limited useful data derived from this study, as detectable residues in fat were mostly outside the validated range of the LC-MS/MS analytical method used (5 - 100 µg/kg in fat), but it added to the evidence that residues were highest in fat samples, with very few detectable residues of ethion in any of the other edible tissues sampled (liver, kidney, muscle). Perirenal fat was sampled in this study.

In the second study (Anon 2), cattle were treated twice, 9 days apart. Groups of 4 animals (1 male and 3 female) were slaughtered on days 5, 10, 15 and 20 post treatment. Samples of muscle, kidney, liver, subcutaneous and perirenal fat were taken from each animal. Muscle samples were taken from the loin (both left and right); samples of subcutaneous fat were taken from along the back line. Residues were undetectable (LOD = 6.5 µg/kg) in all samples except one kidney sample at day 10 and all fat samples at all time points. The withdrawal periods used in the study were only up to 20 days, which is insufficient to cover the duration of approved withdrawal periods for immersion baths, but the depletion profile was comparable to the other data available.

In the third study (Gérez García *et.al.*, 2017), cattle were treated three times, 21 days apart. Groups of 4 animals were slaughtered on days 13, 34, 70, 90, 105 and 117 post final treatment. Samples of muscle (two types; loin and thigh), fat (perirenal fat, plus subcutaneous fat from day 70 onwards), kidney and liver were analysed using GC-ECD, GC-MS and LC-MS/MS. The LOD was 1 µg/kg for all tissues and the LOQs were 5 µg/kg for fat (LC-MS/MS) and muscle, 10 µg/kg for fat (GC-MS), liver and kidney. Samples were analysed in triplicate. In addition, the samples of muscle and fat from slaughter day 34 onwards were re-analysed in a second laboratory. Samples were analysed by LC-MS/MS. The LOQ was 1 µg/kg and the LOD was 0.5 µg/kg for both tissues. No validation data were available for the analytical method used in the second laboratory. The results of analyses of the same samples between the two laboratories were inconsistent, bringing the reliability of these results into question.

It is clear that residues of parent ethion are detectable in fat at higher levels and for a much longer duration after treatment than any other edible tissue sampled. This is consistent with the half-life of the compound.

The Committee noted that the lack of qualitative or quantitative metabolite data is a major omission, and must be addressed before any MRLs can be determined for this substance. The toxicological assessment revealed that at least one metabolite (ethion monoxon) retains significant anticholinesterase activity, and therefore must be accounted for in the residue assessment. In addition, the available data did not identify all the metabolites of concern that lead to the identified reproductive toxicity. One option is to identify and quantify all active ethion metabolites in tissue residues, and include these metabolites along with parent ethion as the marker residue. Alternatively, a single substance can be selected as the marker residue. However, to estimate the toxicological activity of the total ethion residues (including metabolites), knowledge of the marker residue: total residue ratio over time will be required. As such data are not currently available, an accurate assessment of the total toxicological activity of ethion residues (and subsequent residue exposure assessment) cannot be performed.

Analytical methods

The Committee notes that there are analytical methods available for the determination of parent ethion in cattle tissues. However, as the marker residue has not yet been determined, the Committee cannot comment on the suitability of the analytical methods for use in residues control.

Dietary Exposure Assessment

Dietary exposure to ethion may occur through its use as a veterinary drug or its use as a pesticide. While ethion has been assessed by JMPR, no estimate of dietary exposure has been reported from its use as a pesticide.

No dietary exposure assessments were performed for ethion in cattle tissues due to the lack data regarding residue characterisation and total residue concentrations.

Maximum residue limits

In considering MRLs for ethion in cattle edible tissues, the Committee considered the following factors:

- An ADI of 0–0.002 mg/kg bw was established based on the NOAEL of 0.2 mg/kg bw per day in a developmental toxicity study in rats, and using a safety factor of 100.
- An acute reference dose (ARfD) of 0.02 mg/kg bw was established based on the NOAEL of 0.15 mg/kg bw for erythrocyte AChE inhibition in a repeated-dose study in male volunteers, and using a 10-fold intra-species safety factor.
- As the ADI was based on developmental effect and is appreciably lower than the ARfD, there is a potential concern for exposure of 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.
- The residues of concern include all residues derived from ethion, due to the lack of:
 - data relating the toxicological endpoint used to set the ADI to any specific residue or combination of residues, and
 - characterisation of metabolites, either in the laboratory species used in the toxicological studies, or in the edible tissues of the target species, cattle.
- No data regarding total ethion residues in cattle were provided.
- A suitable marker residue could not be determined.

The Committee was unable to recommend MRLs for ethion at this time.

Essential data needed to complete the assessment:

Pharmacokinetics and metabolism and residues depletion in cattle:

In order to enable a determination of a suitable marker residue(s), a metabolism study using radiolabelled ethion in cattle is required. The data should be sufficient to determine:

- The identity of the metabolites produced in cattle;
- The ratios of the parent compound and/or metabolites (i.e., potential marker residues) to the total residues over the residue depletion period in edible tissues (e.g., liver, kidney, muscle and fat).
- Such a study would also provide information on the relative concentration of the target compounds (i.e., marker residue; parent ethion and/or active metabolites) in the various edible tissues of cattle.

A comparison of cattle metabolites to metabolites seen in laboratory species should be conducted to ensure that all residues of toxicological concern produced in cattle have been covered by the available toxicology studies.

Analytical methods:

If it is shown that the marker residue(s) for ethion should be anything other than ethion itself (e.g., ‘sum of ethion + ethion monooxon’), then analytical method(s) that can measure the marker residues in edible tissues (e.g., fat, kidney, liver, muscle) should be developed and validated in accordance with established guidance (CAC/GL71-2009). As there are numerous companies that market ethion formulations, and may perform future residue depletion studies, the use of a common (and suitable) analytical method will be desirable to facilitate comparisons between studies.

Marker residue depletion:

Once a suitable marker residue has been established for ethion, the MR: TRR ratios have been characterised in edible tissues, and a validated analytical method is available for quantifying the marker residue in edible tissues, a non-radiolabelled marker residue depletion study can be conducted. Interested member states should note that the label instructions and usage of ethion products varies significantly. Therefore a single ethion marker residue depletion study may not be suitable for establishing MRLs for all ethion products, due to significant differences in residue depletion and Good Veterinary Practices (label usage and withdrawal times) between formulations. For example, residue data generated from an ethion ear-tag study may not be suitable for establishing MRLs via immersion bath.

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Flumethrin

First draft Prepared by

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Identity

Flumethrin is a pyrethroid acaracide composed of a mixture of two diastereomers (trans-Z1 and trans-Z2, with an approximate ratio 60:40).

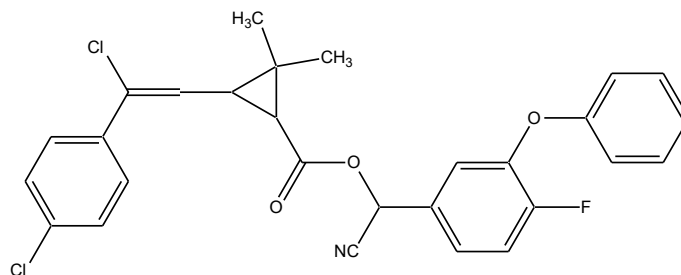
The laboratory studies to determine the physical properties of flumethrin were conducted to GLP.

International Non-proprietary Name (INN): Flumethrin

IUPAC name: (±)-α-cyano-4-fluoro-3-phenoxybenzyl-3-(β,4-dichlorostyryl)-2,2-dimethylcyclopropanecarboxylate or cyano(4-fluoro-3-phenoxyphenyl)methyl 3-[2-chloro-2-(4-chlorophenyl)vinyl]-2,2-dimethylcyclopropanecarboxylate.

Chemical Abstract Service (CAS) No.: 69770-45-2

Structural formula:



Molecular formula: C₂₈H₂₂Cl₂FNO₃

Molecular weight: 510.39 g/mol (pure substance)

Other information on identity and properties

Pure active ingredient: Flumethrin

Appearance: Highly viscous yellowish oil; no characteristic odour.

Solubility (at 20 °C):

In pure water: trans-Z1: 0.1 µg/l; trans-Z2: 0.1 µg/l; sum of trans-Z1 + Z2: 0.2 µg/l

In 1 % NaCl solution: < 0.03 µg/l (both diastereomers)

In water buffered at pH 4 or pH 7: < 0.03 µg/l (both diastereomers)

In water buffered at pH 9: hydrolysis

Table 1 . Solubility in organic solvents

	diastereomer		
	trans-Z1	trans-Z2	trans-Z1 + Z2 (sum)
n-Heptane	11 g/l	8 g/l	19 g/l
Xylene			> 250 g/l
1,2-Dichloroethane			> 250 g/l
2-Propanol	36 g/l	29 g/l	65 g/l
1-Octanol	69 g/l	56 g/l	130 g/l
Polyethyleneglycol			100 - 200 g/l
Acetone			> 250 g/l
Dimethylformamide			> 250 g/l
Acetonitrile			> 250 g/l
Ethylacetate			> 250 g/l
Dimethylsulfoxide			> 250 g/l

Log K_{ow} or Partition Coefficient: Log P_{ow} = 6.2

pH: The substance has no acidic or basic properties in aqueous solutions (the low water solubility makes the substance unsuitable for experimental methods to determine the pH).

Optical rotation: No data were provided.

UVmax: No data were provided.

Stability: On the basis of DTA/TGA measurements carried out in accordance with OECD Guideline No. 113 the substance is thermally stable at ambient temperature under air.

Vapour pressure: The substance has a very low vapour pressure.

Background

Flumethrin is registered in several countries worldwide for the diagnosis and control of varroatosis (aka varroosis) in honey bee colonies. Varroatosis is a disease of honey bees caused by the parasitic mite *Varroa destructor*. The substance can be formulated into a Low Density Polyethylene (LDPE) strip, which is impregnated with 3.6 mg flumethrin (0.5 mg/cm³). 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 to use.

The product is authorised for use in the following countries: Canada, Greece, Ireland, Mexico, New Zealand, Spain, Turkey and the United Kingdom.

Another type of beehive strip, where the flumethrin is impregnated into polyvinyl chloride (PVC), has also been authorised in some member states, which instead of being inserted between the honey combs, is used as a gate at the entrance of the beehive.

Residues in food and their evaluation

Conditions of use

Between-comb strips:

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 (New Zealand only; this is not stated directly in other regions, but it is implied).

Beehive gates:

All colonies located on the same apiary should be treated simultaneously.

The product is intended to be used as part of an integrated varroa control programme.

As an effective method to reduce the risk of resistance selection, flumethrin – as for other acaricides – should not be used in consecutive years. Instead, strict rotation with products containing active substances from other chemical classes should be applied. Depending on the regional resistance situation, a longer treatment break than one year may be necessary. As flumethrin and tau-fluvalinate belong to the same class, they are not suitable for rotation with each other.

Inappropriate use of the product could result in an increased risk of resistance development and could ultimately result in ineffective therapy and colony losses.

Flight activity is necessary for exposure to the active substance. In case of prolonged periods of low flight activity, e.g., due to unfavourable weather conditions, efficacy may be reduced.

The beehive gates should not be reused.

Dosage

Between-comb strips:

The usual dose recommended in all regions where the between-comb product is authorised is 4 strips per chamber in developed colonies, or 2 strips per chamber in young colonies. The strips are reported to contain 3.6 mg flumethrin each (0.5 mg/cm³). The strips are suspended in the spaces between the combs in the central brood rearing area. The strips should remain in the colony for 24 h (diagnosis) or for 4 to 8 weeks (treatment), although the most common recommendation is for 6 weeks.

Beehive gates:

The recommended dose for the beehive gate strips (275 mg flumethrin per strip) is 2 strips per beehive, fitted to the entrance of the hive, so that the bees are forced to use the holes that are 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.

Pharmacokinetics and metabolism

Data on mammalian pharmacokinetic and metabolism are provided in the toxicology submission.

Pharmacokinetics in laboratory animals

When groups of rats were given 1 mg/kg bw $^{14}\text{C}[\text{Cl}]$ -flumethrin PO in 5 % aqueous Cremophor vehicle, 68 % of the radioactivity was eliminated in the faeces, and around 2 % in the urine, during the first 24 h.

Conversely, after PO administration of 1 mg/kg bw $^{14}\text{C}[\text{F}]$ -flumethrin, 45 % of the radioactivity was eliminated in urine and the rest in the faeces. Females appeared to absorb a considerably higher percentage of the dose than males after oral administration.

The plasma elimination half-lives of $^{14}\text{C}[\text{Cl}]$ - and $^{14}\text{C}[\text{F}]$ -flumethrin in PO-dosed rats were approximately 160 h and 8 h, respectively. No information on the nature of the metabolites derived from the acid moiety (which was radiolabelled in the chlorophenyl ring), which remained in the blood of treated rats for prolonged periods, are available.

Repeated daily PO dosing of 1 mg/kg bw $^{14}\text{C}[\text{Cl}]$ -flumethrin to 8 male rats resulted in an accumulation of radioactivity in the plasma.

After PO administration of 1 mg/kg bw ^{14}C -flumethrin to rats of both sexes, residues concentrations were highest in plasma (V_{ss} approximately 0.4 l/kg), and most of the radioactivity remained in the stomach for up to 312 h after administration.

Pharmacokinetics in Food-producing Animals (bees/honey)

Pharmacokinetic studies in beehives or honey are not applicable.

When bee colonies were exposed to the flumethrin-impregnated strips in the spring, pre-winter and during the nectar flow period, the flumethrin concentration in honey was found to be < LOD (1-2 $\mu\text{g/kg}$). The content in wax from the nearby combs was 30, 40 and 90 $\mu\text{g/kg}$, respectively. The highest concentration of flumethrin detected in beeswax was 130 $\mu\text{g/kg}$, found in a sample from a hive treated during the honey flow period.

Transfer of flumethrin from beeswax to honey was negligible, but residues in beeswax may accumulate if the wax is reused over several years. Residues of up to 61 $\mu\text{g/kg}$ were found in wax from hives that had been treated annually for approximately 10 years.

In supervised residue trials for beehive gates used according to proposed label, with 2 strips (275 mg/strip) applied at the entrance of the hive, residues of flumethrin in honey were <LOQ

(<3 µg/kg). Residues of flumethrin in wax were in the range of <25 – 119 µg/kg. ***Metabolism in Laboratory Animals***

Rats

In rats, flumethrin is metabolised by hydrolysis of its central ester bond to form flumethrin acid and 3-phenol-4-fluorobenzyl alcohol. Flumethrin acid is then conjugated with glucuronic acid to form the glucuronide. 3-phenol-4-fluorobenzyl alcohol is not detected in the rat, but is oxidised to 4-fluoro-3-phenoxybenzoic acid and then to 4-fluoro-3-(4-hydroxyphenoxy)benzoic acid, both of which form glycine conjugates.

Metabolism in Food Producing Animals (bees/honey)

There is no known metabolism of the substance by the bees; the majority of the residues end up in the beeswax and honey, where no biotransformation can take place. Therefore, the only way of reducing the concentration of flumethrin in the honey is via a degradation process. However, investigations into the stability of residues of flumethrin in honey stored at room temperature have shown that no significant decrease in concentration was observed over a storage period of 9 months. This demonstrates that flumethrin does not degrade in honey. It is noted that the Committee has not evaluated any data regarding the disposition of flumethrin in other commodities derived from beehives, such as propolis, royal jelly, etc.

Comparative Metabolism

Since flumethrin is not metabolised when used in beehives, no comparative metabolism can be determined.

Residue depletion studies in honey

Radiolabelled residue depletion studies

No radiolabelled residues depletion study has been conducted or provided.

Residues depletion studies with non-radiolabelled drug

Between-comb strips:

Six non-GLP compliant residues depletion studies of flumethrin in honey produced by honeybees were conducted in various locations in Germany and the UK. All studies used Bayvarol strips, which are LDPE strips impregnated with 3.6 mg flumethrin per strip.

In one study, 24 honeybee colonies were treated with 4 strips each, corresponding to a total dose of 14.4 mg flumethrin, for a duration of 6 months, over the winter period. One sample was analysed, no residues were detected (LOQ = 3 µg/kg; LOD = 1 µg/kg). At another site, 12 honeybee colonies were treated for 6 weeks with 4 strips per hive, from the beginning of September to the middle of October, over 2 years. Four samples were analysed, no residues were detected (LOQ = 3 µg/kg; LOD = 1 µg/kg).

In the second study, honeybee colonies were treated with 4 strips per brood chamber for six weeks, after the honey harvest. One sample was taken, during the spring honey flow. The results of the analysis showed no detectable residues of flumethrin in the honey (LOQ = 3 µg/kg; LOD = 1 µg/kg).

In the third study, six honeybee colonies were treated for 6 weeks, from September to October, with 4 strips per frame. Six samples were taken in June the next year and combined in pairs to form three samples for analysis. The results of analysis from all samples showed no residues above the Limit of Quantification ($\text{LOQ} = 2 \mu\text{g/kg}$).

In the fourth study, six honeybee colonies were treated for 5 months from October to March with 4 strips per frame. Six samples of freshly capped honeycomb were taken in June of the following year, after the early nectar flow. The results of analysis from all samples showed no residues above the Limit of Quantification ($\text{LOQ} = 2 \mu\text{g/kg}$).

In the fifth study, seven honeybee colonies were treated from early March to mid-April with 4 strips per frame. At the end of the treatment period, these combs were labelled and suspended in the honey chamber until the last brood had hatched and fresh honey had been inserted and capped. The results of analysis from all samples showed no residues above the Limit of Quantification ($\text{LOQ} = 2 \mu\text{g/kg}$).

In the final non-GLP study, four colonies of honeybees were treated for 4 months from May to September, during the honey flow period. The entire honey harvest of each treated colony was centrifuged separately in the last week of August. The results of analysis from all samples showed no residues above the Limit of Quantification ($\text{LOQ} = 2 \mu\text{g/kg}$).

Table 2. Summary of residues trials using between-comb strips

Honey Trial Country, year	Formulation and use rate	Treatment			Treatment Period	Time of sample collection		Reference
		No colonies	No of samples	Duration (weeks)		sample collection	Flumethrin residue (µg/kg)	
Lindlar, Germany 1992-1993	Bayvarol strips 3.6 mg/strip 4 strips/hive	24	1	23	12 Oct 1992- 08 Mar 1993	15 Jun 1993	≤1	Krebber, R., 1994a Report RA-
Leverkusen, Germany 1991-1992	Bayvarol strips 3.6 mg/strip 4 strips/hive	12	4	6	early Sept- mid Oct 1991 early Sept- mid Oct 1992	1993 (end of fruit and dandelion flowering)	≤ x 4	158/94 Bayer 014313 Ref:
UK 1992-1993	Bayvarol strips 3.6 mg/strip Use rate not reported	Not reported	1	Not reported	Not reported	Spring 1993	≤1	Krebber, R., 1994b Report RA- 233/94 Bayer 014338 Ref:
Rhineland, Germany 1987-88	Bayvarol strips 3.6 mg/strip 4 strips/hive	6	3 (each sample from 2 colonies)	6	early Sept - mid- Oct 1987 (pre-winter storage period)	June 1988 (after early nectar flow)	≤ 2 x 3	Riegner, K., Krieger, K., 1990a Report 90/13521 Bayer 015989 Ref:

Honey Trial Country, year	Treatment		No of colonies	No of samples	Duration (weeks)	Treatment Period	Time of sample collection	Flumethrin residue (µg/kg)	Reference
	Formulation and use rate								
Bergische Land, Germany 1987-1988	Bayvarol strips 3.6 mg/strip 4 strips/hive	6	6	18	late Oct 1987 – mid Mar 1988	June 1988 (after early nectar flow)	≤ 2 x 6	Riegner, K., Krieger, K., 1990b Report No. 90/13522 Bayer Ref: 015990	
Taunus, Germany 1985-1986	Bayvarol strips 3.6 mg/strip 4 strips/hive	7	9	Not reported	early Mar - mid Apr 1986	Not reported	≤ 2 x 9	Riegner, K., Krieger, K., 1990c Report No. 90/13523 Bayer Ref: 015991	
Bergische Land, Germany 1988	Bayvarol strips 3.6 mg/strip 4 strips/hive	4	4	20	May 1988 - Sept 1988 (during nectar flow)	August 1988 (final week)	≤ 2 x 4	Riegner, K., Krieger, K., 1990d Report No. 90/13524 Bayer Ref: 015992	

Description of individual residue trials in honey using between-comb strips**Reference AH ID 014313en.pdf (Report No. 158/94)**

Residues of Flumethrin in Honey after Administration of Bayvarol Strips to Honeybee Colonies in Germany; Project number P65335008 (Krebber, 1994a).

At one site, in Lindlar, North Rhine-Westphalia, Germany, 24 colonies of honeybees were treated with 4 LDPE strips impregnated with flumethrin. Treatment commenced on 12th October 1992 and was completed (the strips were removed) on 8th March 1993, so these colonies were exposed to the product for 6 months, over winter. The honey was sampled on 15th June 1993 and stored at 4 °C until analysis. One sample was analysed.

At another site, in Leverkusen, North Rhine-Westphalia, Germany, 12 honeybee colonies were treated for 6 weeks with 4 strips per hive from the beginning of September to the middle of October over 2 years (1991 & 1992). Honey samples were taken in 1993, at the end of fruit and dandelion flowering (exact dates not stated). Four samples were analysed.

All samples were analysed using method RA-654/93 (HPLC-UV).

Table 3. Recovery from honey fortified with 3 µg/kg flumethrin

Recovery values (%) n = 4				Mean (%)	Relative Standard Deviation (RSD %)
93	93	83	80	87.25	7.7

The results of the analyses showed no detectable residues of flumethrin in the honey from the treated colonies (LOQ = 3 µg/kg; LOD = 1 µg/kg).

Reference AH ID 014338en.pdf (Report No. RA-233/94)

Residues of Flumethrin in Honey after Administration of Bayvarol Strips to Honeybee Colonies in Great Britain; Project number P65335009, Report number RA-233/94 (Krebber, 1994b).

Beehives in Great Britain were treated as recommended in the instructions for use. One sample was taken during the spring honey flow of 1993. No further information was provided.

The sample was analysed using method RA-654/93 (HPLC-UV).

The results of the analysis showed no detectable residues of flumethrin in the honey (LOQ = 3 µg/kg; LOD = 1 µg/kg).

Reference AH ID 015989en.pdf (Doc No. 90/13521)

Residues of Flumethrin in Honey Following the Use of Bayvarol Strips during the Pre-Winter Storage Period (Riegner & Krieger, 1990a)

Six commercial bee colonies belonging to a single beekeeper in the Rhineland, Germany were treated for 6 weeks, from early September to mid-October 1987, with 4 strips per frame (each frame contained 8-10 Freudenstein combs). Two strips were suspended in each of two gaps between the central combs of the brood nest, so that the bees could crawl over both sides.

In June 1988, after the early nectar flow, one freshly capped honeycomb from the honey chamber of each colony was removed and centrifuged. The samples obtained were stored at 4 °C until preparation for analysis.

Equal weights of samples 1 & 2, 3 & 4, and 5 & 6, respectively, were combined to give three samples, which were analysed on 12th August 1987, using method RA-197. The report number of the analysis part is RA-1104/RGK046.

The results of analysis from all samples showed no residues above the Limit of Quantification (LOQ = 2 µg/kg).

Reference AH ID 015990en.pdf (Doc No. 90/13522)

Residues of Flumethrin in Honey Following the Use of Bayvarol Strips During the Winter (Riegner & Krieger, 1990b).

Six experimental colonies of honeybees in Bergische Land, North Rhine-Westphalia, Germany, were treated for 5 months from late October 1987 to mid-March 1988 with 4 strips per frame. Two strips were suspended in each of two gaps between the central combs of the brood nest, so that the bees could crawl over both sides. Six samples of freshly capped honeycomb were taken in June of the following year, after the early nectar flow.

Samples 1 & 2, 3 & 4, and 5 & 6, respectively, were combined to give three samples, which were analysed on 21/22 Jun 1988, using method RA-197. The report number of the analysis part is RA-1106/RGK048.

The results of analysis from all samples showed no residues above the LOQ (LOQ = 2 µg/kg).

Reference AH ID 015991en.pdf (Doc number 90/13523)

Residues of Flumethrin in Honey Following the Use of Bayvarol Strips in Spring (Doc number 90/13523, Riegner & Krieger, 1990c)

Seven experimental honey bee colonies in Taunus, Hesse, Germany, were treated from early March to mid-April 1986 with 4 strips per frame (10 Zander combs per frame).

Two strips were suspended between combs 3 & 4 and between combs 7 & 8, so that the bees could crawl over both sides.

At the end of the treatment period, these combs were labelled and suspended in the honey chamber until the last brood had hatched and fresh honey had been inserted and capped.

The honey from combs 3 & 4 and combs 7 & 8 was centrifuged and analysed separately.

In order to compare two colonies (5 and 21), honey from the two combs furthest away from the strips (1 & 10) was centrifuged and analysed.

The samples were analysed in December 1985 using method RA-197. The report number of the analysis part is RA-273.

The results of analysis from all samples showed no residues above the Limit of Quantification (LOQ = 2 µg/kg).

Reference AH ID 015992en.pdf (Doc number 90/13524)

Residues of Flumethrin in Honey Following the Use of Bayvarol Strips During the Nectar Flow Period (Doc number 90/13524, Riegner & Krieger, 1990d)

Four experimental colonies of honey bees in Bergische Land, North Rhine-Westphalia, Germany, were treated from May to September (4 months) 1988, with 4 strips per brood frame (each containing 11 DN combs).

The strips were inserted into the honey chamber when the colony swarmed in the first week of May, and were suspended in the central gaps between the combs, so that the bees could crawl over both sides.

The entire honey harvest of each treated colony was centrifuged separately in the last week of August.

The samples were stored at 4 °C until being prepared for analysis. The samples were analysed on 14th October 1988, using method RA-197. The report number of the analysis part is RA-1110/RGK053.

The results of analysis from all samples showed no residues above the LOQ (LOQ = 2 µg/kg).

Table 4. Summary of residues studies using beehive gates

Honey Trial	Formulation and use rate	No of colonies	No of samples	Treatment	Duration (weeks)	Treatment Period	Time of sample collection	Flumethrin residue ($\mu\text{g/kg}$)	Reference
Apiary A1 63571 Gelnhausen, Germany 2014-2015	PolyVar Yellow strips 275 mg/strip 2 strips/hive	7	1		17	13 Aug 2014 – 11 Dec 2014	21 May 2015	≤ 3	Kreber, R. and Hoffend, J., 2015 Report MR-15/183 Bayer Ref: 41149
Apiary A2 63543 Neuberg, Germany 2014-2015	PolyVar Yellow strips 275 mg/strip 2 strips/hive	6	1		17	13 Aug 2014 – 11 Dec 2014	21 May 2015	≤ 3	Altreuther, G., 2017, Supplementary information to study 41149.
Apiary A3 55131 Mainz, Germany 2014-2015	PolyVar Yellow strips 275 mg/strip 2 strips/hive	6	1		18	14 Aug 2014 – 15 Dec 2014	25 May 2015	≤ 3	
Apiary A4 63526 Erlensee, Germany 2014-2015	PolyVar Yellow strips 275 mg/strip 2 strips/hive	13	1		17	13 Aug 2014 – 11 Dec 2014	20 May 2015	≤ 3	
Apiary B1 6708 PB Wageningen, The Netherlands 2014-2015	PolyVar Yellow strips 275 mg/strip 2 strips/hive	6	1		17			≤ 3	
Apiary B2 5386 KR Geffen, The Netherlands 2014-2015	PolyVar Yellow strips 275 mg/strip 2 strips/hive	5	1		17	20 Aug 2014 – 16 Dec 2014	16 Jun 2015	≤ 3	

Description of residue trials in honey using Beehive gate strips.**Reference ID41149:**

Determination of flumethrin in honey and wax. Report No.: MR-15/183. Study Number: P673155038 (Study No. 201394), (Krebber & Hoffend, 2015).

The applicant provided one GLP-compliant field study conducted in beehives, using beehive gate strips: Honeycombs were obtained from different test sites in Germany (4 apiaries), Hungary (2 apiaries), Spain (2 apiaries) and The Netherlands (3 apiaries) after application of Polyvar Yellow (flumethrin bee-hive gate). Both honey and beeswax were sampled and analysed.

Thirty-two honeycomb samples from four apiaries in Germany, 14 samples from two apiaries in Hungary, 12 samples from two apiaries in Spain and 18 samples from three apiaries in The Netherlands were analysed.

The maximum application time at hive entrance of 4 months is covered by data from Germany (122 or 120 days) and the Netherlands (119 days). In the other regions, application time was 92 days (Spain) and 102 days (Hungary).

For separation of honey and wax, honey-filled wax cells were scraped off from representative areas of the upper third from both sides of the collected honey combs and transferred to a plastic bag. Each of these sample bags was kept at 40 °C until wax has floated and separated from the honey. This procedure took up to five days. Then, one edge of the bottom of each plastic bag was cut and the honey was collected in a beaker. Aliquots of the samples were filled into glass vials. The remaining mixture in the bag was transferred to a sieve and the remaining honey was washed off thoroughly with water. The pure wax was then transferred to a glass dish and kept at 40 °C until it was dry and transferred to a new plastic bag for storage.

For residue analysis the preparation of honey and wax pooled samples from all hives of one apiary was necessary. Therefore, equal amounts of the previously purified honey samples were combined and homogenized by stirring at 40 °C. Wax samples were melted at around 63 °C, homogenised and purified from contamination by skimming off or passing it again through a sieve.

The honey and wax samples were analysed for concentrations of flumethrin based using a validated LC-MS/MS method.

Residue concentrations in honey were below the limit of quantification of 3 µg/kg and also below the lowest calibration standard which corresponds to 1 µg/kg.

Residue depletion studies in beeswax***Between-comb strips***

Five non-GLP compliant residues depletion studies of flumethrin in beeswax produced by honeybees were conducted in various locations in Germany and Switzerland. All studies used between-comb strips, impregnated with 3.6 mg flumethrin per strip.

In the first study, 15 beehives were treated as per product instructions (4 strips per brood chamber for six weeks, after the honey harvest) in three Swiss cantons. Thirteen samples of beeswax were analysed for flumethrin and residues were found in the range $< 26 \mu\text{g/kg}$ to $176 \mu\text{g/kg}$. Mean residues were around $50 \mu\text{g/kg}$. The LOQ for the analytical method used was $26 \mu\text{g/kg}$.

In the second study, an unreported number of beehives were treated with ten times the recommended number of strips (40 strips per hive). The analysis of four samples was reported and the results were in the range $70 - 146 \mu\text{g/kg}$; mean = $106 \mu\text{g/kg}$.

In the third study, two beehives were treated with 4 strips per chamber for 6 weeks, just before the start of honey flow. Two samples per frame were analysed. Residues in the range < 15 to $40 \mu\text{g/kg}$ were reported. The LOQ of the analytical method used was $15 \mu\text{g/kg}$.

In the fourth study, six beehives were treated with 4 strips per brood chamber for 6 weeks, from September to October, after the honey harvest. The following year, samples were taken in June, after the early honey flow. One honeycomb was taken from each hive and combined with one from another beehive to form three samples, which were then analysed for flumethrin. The results of the analyses showed residues in the range of $< 20 - 50 \mu\text{g/kg}$.

In the final study, four beehives were treated with 4 strips per brood chamber for six months, during the honey flow period. The strips were inserted in May and removed in September. Two combs from each hive that had been in the brood chamber were moved to the honey chamber in mid-August until the brood hatched. These combs were sampled and analysed. The results were in the range $30 - 130 \mu\text{g/kg}$.

Beehive gates

With the beehive gates, four supervised trials were carried out on wax in 11 apiaries in Europe during 2014-2015. All trials were conducted according to proposed label with 2 strips applied at the entrance of each hive. Each apiary consisted of multiple colonies.

Residues of flumethrin in wax were in the range of $<0.025 - 0.119 \text{ mg/kg}$. The results of the trials are presented. Samples were analysed using an HPLC/MS/MS method with an LOQ of $25 \mu\text{g/kg}$. The mean procedural recovery was 99 % (fortification range $25-250 \mu\text{g/kg}$, $n = 18$, RSD = 6.2 %).

Table 5. Summary of residues in beeswax

Beeswax Trial	Treatment	No of colonies	No of samples	Duration (weeks)	Treatment Period	Time of sample collection	Flumethrin residue (µg/kg)
Country, year	Formulation and use rate						
6482 Seedorf, Uri, Switzerland 1992-1993	Bayvarol strips 3.6 mg/strip "Following instructions for use"	Not reported	1	Not reported	1992 and 1993	1993	53
6484 Wassen, Uri, Switzerland 1992-1993	Bayvarol strips 3.6 mg/strip Use rate not reported	Not reported	1	Not reported	1992 and 1993	1993	26
1510 Moudon, Waadt, Switzerland 1992-1993	Bayvarol strips 3.6 mg/strip Use rate not reported	Not reported	1	Not reported	1992 and 1993	1993	≤26
1812 Ecoteaux, Waadt, Switzerland 1992-1993	Bayvarol strips 3.6 mg/strip Use rate not reported	Not reported	1	Not reported	1992 and 1993	1993	≤26
1142 Pampigny, Waadt, Switzerland 1992-1993	Bayvarol strips 3.6 mg/strip Use rate not reported	Not reported	1	Not reported	1992 and 1993	1993	26
1142 Eclepéns, Waadt, Switzerland 1992-1993	Bayvarol strips 3.6 mg/strip Use rate not reported	Not reported	1	Not reported	1992 and 1993	1993	≤26
1142 Eclepéns, Waadt, Switzerland 1992-1993	Bayvarol strips 3.6 mg/strip Use rate not reported	Not reported	1	Not reported	1992 and 1993	1993	72
1110 Morges, Waadt, Switzerland 1992-1993	Bayvarol strips 3.6 mg/strip Use rate not reported	Not reported	1	Not reported	1992 and 1993	1993	55

Beeswax		Treatment				Time of	
Trial	Formulation and use rate	No of colonies	No of samples	Duration (weeks)	Treatment Period	sample collection	Flumethrin residue (µg/kg)
1073 Savigny, Waadt, Switzerland	Bayvarol strips 3.6 mg/strip Use rate not reported	Not reported	1	Not reported	1992 and 1993	1993	46
2300 La Chaux-de-Fonds, Neuenburg, Switzerland	Bayvarol strips 3.6 mg/strip Use rate not reported	Not reported	1	Not reported	1992 and 1993	1993	68
2300 La Chaux-de-Fonds, Neuenburg, Switzerland	Bayvarol strips 3.6 mg/strip Use rate not reported	Not reported	1	Not reported	1992 and 1993	1993	≤ 26
2300 La Chaux-de-Fonds, Neuenburg, Switzerland	Bayvarol strips 3.6 mg/strip Use rate not reported	Not reported	1	Not reported	1992 and 1993	1993	39
2300 La Chaux-de-Fonds, Neuenburg, Switzerland	Bayvarol strips 3.6 mg/strip Use rate not reported	Not reported	1	Not reported	1992 and 1993	1993	176
Freiburg, Germany 1991	Bayvarol strips 3.6 mg/strip 40 strips/hive (10 x GVP rate)	4	4	4	28 Jun 1991 – 23 Jul 1991	13 Aug 1991	146 105 70 103
Taunus, Germany 1986	Bayvarol strips 3.6 mg/strip 4 strips/hive	2	4	6	early Mar-mid Apr 1986 (before nectar flow)	mid Apr 1986	17, <15 40, 15

Beeswax Trial	Treatment Formulation and use rate	No of colonies	No of samples	Duration (weeks)	Treatment Period	Time of sample collection	Flumethrin residue (µg/kg)
Country, year							
Rhineland, Germany 1987-1988	Bayvarol strips 3.6 mg/strip 4 strips/hive	6	3	6	Early Sept – mid Oct 1987	June 1988 after early nectar flow)	≤ 20 4 0 5 0
Bergische Land, Germany 1988	Bayvarol strips 3.6 mg/strip 4 strips/hive	4	4	20	May – Sept 1988	Sept 1988	130 100 30 100
Apiary A1 63571 Gelnhausen, Germany	PolyVar Yellow strips 275 mg/strip 2 strips/hive	7	1	17	13 Aug 2014 – 11 Dec 2014	21 May 2015	119
2014-2015							
Apiary A2 63543 Neuberg, Germany	PolyVar Yellow strips 275 mg/strip 2 strips/hive	6	1	17	13 Aug 2014 – 11 Dec 2014	21 May 2015	≤ 25
2014-2015							
Apiary A3 55131 Mainz, Germany	PolyVar Yellow strips 275 mg/strip 2 strips/hive	6	1	18	14 Aug 2014 – 15 Dec 2014	25 May 2015	93
2014-2015							
Apiary A4 63526 Erlensee, Germany	PolyVar Yellow strips 275 mg/strip 2 strips/hive	13	1	17	13 Aug 2014 – 11 Dec 2014	20 May 2015	≤ 25
2014-2015							

Description of individual residue trials in wax using between-comb strips**Reference AH ID 014583en.pdf**

Residues of Flumethrin in Beeswax after Administration of Bayvarol Strips to Honeybee Colonies in Switzerland (Krebber, 1994 – Report No. RA-126/94; Project No. P65345009)

Honeybee colonies in the Swiss cantons Neuenburg, Uri and Waadt were treated with Bayvarol strips according to the instructions for use in the years 1992 and 1993. Wax samples were taken in 1993 and analysed for residues of flumethrin (in July 1994).

The samples were analysed using method RA-654/93.

Table 6. Concurrent QC recoveries:

Fortification level (µg/kg)	Recovered (%)	Mean value (%)
26*	92 62 77 104	84
100	85 74	80

*Determined as LOQ.

Analyses were performed from July 12 to July 26, 1994. The concentrations of residue were in the range of < LOQ (26 µg/kg) to 176 µg/kg. The results were not corrected with respect to the recovery rate.

Reference AH ID 014714en.pdf

Residues of Flumethrin in Beeswax after Treatment with a Tenfold Overdose of Bayvarol Strips to Honeybee Colonies in Germany (Krebber 1994, Report No. 286/94; Project No. P65335010).

Beehives in Freiburg, Germany were treated with a 10 x overdose of Bayvarol Strips (40 strips per hive) from 28th June 1991 to 23rd July 1991. Four 10 g samples of wax were taken on 13th August 1991 and analysed for residues of flumethrin (in August 1994).

The samples were analysed using method RA-654/93.

Table 7. Concurrent QC recoveries:

Fortification level (µg/kg)	Recovered (%)	Mean value (%)
26*	92 62 77 104 85	84
100	85 74 81	80

*Determined as LOQ.

The concentrations were between 103 and 146 µg/kg. Analyses were performed from 2nd to 4th August, 1994. The results were not corrected with respect to the recovery rate.

No raw data were provided.

Reference AH ID 015993en.pdf

Residues of Flumethrin in Beeswax Following the Use of Bayvarol Strips in Spring. (Riegner & Krieger 1990; Doc No. 90/13518)

Two experimental bee colonies in the Taunus region of Germany were treated with 4 strips per frame (10 Zander combs per frame) for 6 weeks, from early March to Mid-April 1986, immediately before the start of the nectar flow period. Two strips each were suspended between combs 3 and 4, and 7 and 8, respectively, so that the bees could crawl over both sides of the strips.

At the end of the treatment period, combs 3 and 8, and 4 and 7, respectively, were combined to form two samples per frame. Samples were analysed using method RA-197. The report number was RA-273. The LOQ for the method was stated as being 15 µg/kg wax.

Samples were analysed on 6th March 1986. One concurrent QC at 200 µg/kg was analysed (n=5).

The concentrations were between <LOQ and 40 µg/kg (corrected for recovery of 62 %).

Reference AH ID 015994en.pdf

Residues of Flumethrin in Beeswax Following the Use of Bayvarol Strips during the Pre-winter Storage Period (Riegner & Krieger, 1990; Doc No. 90/13519).

Six commercial bee colonies in the Rhineland, Germany, were each treated for 6 weeks with 4 strips per frame (8-10 Freudenstein combs per frame), from early September to mid-October 1987. The strips were suspended between the central combs of the brood nest, so that the bees could crawl over both sides of the strips.

In June the following year, after early nectar flow, one freshly capped honeycomb was removed from the honey chamber of each colony and centrifuged (to separate the honey from the comb). The comb samples were stored at 4 °C until preparation for analysis. Equal parts (w/w) of samples 1 & 2, 3 & 4 and 5 & 6, respectively, were combined to form three samples, which were then analysed using method RA-197 (report no RA-1104/RGK046, not provided).

Samples were analysed on 14th and 22nd October 1987

The concentrations were between < LOQ and 50 µg/kg (corrected for recovery of 62 %).

Concurrent QC samples were also analysed (n = 1 each) 473 µg/kg on the 14th, and 472 µg/kg on the 22nd. The LOQ was reported as 20 µg/kg.

Reference AH ID 015995en.pdf

Residues of Flumethrin in Beeswax Following the Use of Bayvarol Strips during the Nectar Flow Period (Riegner & Krieger, 1990; Doc No. 90/13520)

Four experimental colonies of domestic honey bees in Bergische Land were treated with 4 Bayvarol strips per brood frame (11 DN combs per frame) from May to September 1988. The strips were inserted around the honey chamber when the colony swarmed in the first week of May. The strips were suspended in the central passages of the brood chamber, between the combs, so that the bees could crawl over both sides of the strips. Two combs that had been in

the brood chamber until mid-August were moved to the honey chamber until the brood hatched. These combs were removed and stored at 4 °C until being prepared for analysis.

The residues analysis was performed using method RA-197 and the procedure was recorded under RA-1113/RGK073. The analyses took place on 9th December 1988 (samples B-2, B-3, & B-4), and 3rd December 1988 (sample B-5).

The results of the analyses were between 30 and 130 µg/kg (corrected for recovery of 62 %).

Concurrent QC samples (n=2) were analysed with each analytical run, spiked at 253 ppb. The mean results of the test samples were compared to the mean of two QC samples to determine the concentration of flumethrin in the test samples.

Description of individual residue trials in wax using beehive gates

Four supervised trials were carried out using beehive gates, in Germany (4 apiaries), Hungary (2 apiaries), Spain (2 apiaries) and the Netherlands (3 apiaries) during 2014-2015. All trials were conducted according to proposed label for PolyVar Yellow, with 2 strips applied at the entrance of each hive. Each apiary consisted of multiple colonies. Honeycomb samples were collected from each colony and stored at either ambient temperature or frozen prior to shipment to the analytical laboratory. Wax was separated from the honeycombs and pooled to give one wax sample per apiary. The pooled wax samples were stored frozen at < 18 °C prior to analysis.

Samples were analysed using method an HPLC/MS/MS method (01462) with an LOQ of 25 µg/kg. The mean procedural recovery was 99 % (fortification range 25-250 µg/kg, n = 18, RSD = 6.2 %).

Prior to separation of the wax from the honeycomb, samples were stored at ambient temperature for up to 56 days or frozen for up to 72 days. After separation from the honeycomb, wax samples were stored frozen at < 18 °C for up to 44 days before analysis. In the trials, therefore, all wax samples were analysed within 3.5 months after collection.

Residues of flumethrin in wax were in the range of < 25 – 119 µg/kg.

Methods of analysis for residues in honey and beeswax

Table 8. Analytical Methods

Reference Number	Author(s)	Year	Study Title
016001	Riegner, K.	1986	Analytical method to determine the content of Bayvarol (FCR-1622) in honey and beeswax. Report No. RA-197 Bayer Ref: 016001 Non-GLP; Unpublished 25 Mar 1986
013910	Heukamp, U.	1993	Analytical method for the determination of Bayvarol (active ingredient flumethrin) in honey and wax. Report No. RA- 411/92

			Bayer Ref: 013910 Non-GLP; Unpublished 02 Apr 1993
016000	Heukamp, U., 1993 Krebber, R.		Method for determining the residue of flumethrin in bees' honey and wax. Report No. RA-654/93 Bayer Ref: 016000 Non-GLP; Unpublished 02 Nov 1993
041150	Krebber, R., 2015 Hoffend, J., Anjard, O.		Analytical method for the determination of flumethrin in bees honey and wax by LC-MS/MS Report No. MR-15/101, Bayer Ref: 041150 Non-GLP; Unpublished 03 Dec 2015 Also referred to as 'Enforcement Method' Method 01462

Summary of Method Validation Data

Table 9. Methods based on HPLC/UV

Matrix	Analyte/ Method	Fortification µg/kg	n	Range Recovery (%)	Mean recovery (%)	% RSD	Method	Reference
Honey	Flumethrin	3.1	2	61-63	62	-	RA-197	Riegner, 1986, Report No. RA-197, Bayer Ref: 016001
		3.8	2	54-74	64	-		
		4.4	3	58-70	63	10		
Honey	Flumethrin	3	5	72-76	74	2	00317 and 00339	Heukamp, 1993, Report No. RA-411/92, Bayer Ref: 013910 Heukamp and Krebber, 1993, Report No. RA-654/93, Bayer Ref: 016000
		13	5	85-88	87	1		
		85	5	83-89	86	3		
Wax	Flumethrin	25	2	58-66	62	-	RA-197	Riegner, 1986, Report No. RA-197, Bayer Ref: 016001
		50	2	53-69	61	-		
		100	3	58-69	63	9		
Wax	Flumethrin	26	5	54-65	60	7	00317 and 00339	Heukamp, 1993, Report No. RA-411/92, Bayer Ref: 013910 Heukamp and Krebber, 1993, Report No. RA-654/93, Bayer Ref: 016000
		51	5	65-90	79	13		
		102	5	61-96	76	19		

Table 10. Methods based on LC-MS/MS

Matrix	Analyte	Fortification Level (µg/kg)	Range Recovery (%)	n	Mean (%)	RSD (%)	Reference
Honey (quantification m/z 527 → m/z 267)	Flumethrin (LC- MS/MS)	3	78-97	6	85	8.6	Method 01462 Krebber, Hoffend and Anjard, 2015, Report No. MR- 15/101, Bayer Ref: 041150
		6	78-90	6	86	4.9	
		30	77-90	6	83	5.3	
		Overall	77-97	18	85	6.4	
Honey (confirmation m/z 527 → m/z 239)	Flumethrin (LC- MS/MS)	3	80-98	6	88	8.8	
		6	76-94	6	86	6.7	
		30	79-92	6	84	5.8	
		Overall	76-98	18	86	7.1	
Wax (quantification m/z 527 → m/z 267)	Flumethrin (LC- MS/MS)	25	87-103	6	96	6.7	
		50	98-104	6	101	2.1	
		250	87-108	6	101	7.4	
		Overall	87-108	18	99	6.2	
Wax (confirmation m/z 527 → m/z 239)	Flumethrin (LC- MS/MS)	25	66-96	6	83	19	
		50	87-113	6	99	9.4	
		250	85-103	6	96	7.0	
		Overall	66-113	18	92	13	

Method RA-654/93 summary:**Honey:**

Flumethrin was extracted from honey with a mixture of toluene, dichloromethane and methanol. After evaporation to dryness, the residue was re-dissolved in ethylacetate/cyclohexane. Further clean-up was performed by gel permeation chromatography on silica gel, with an elution mixture of n-hexane and dichloromethane. Flumethrin was determined by HPLC-UV.

Beeswax:

Flumethrin was extracted from 10 g wax by boiling with a mixture of 2-propanol and methanol. After addition of water and cooling in an ice bath, the suspension was filtered and the extract was evaporated to a small volume. Further clean-up was performed by partitioning with a mixture of cyclohexane and ethyl acetate followed by column chromatography on silica gel. Flumethrin was determined by HPLC with UV detection. The limit of quantification was 26 µg/kg.

Table 11. Recovery from honey fortified with 3 µg/kg flumethrin

Recovery values (%) n = 4				Mean (%)	Relative Standard Deviation (RSD %)
93	93	83	80	87.25	7.7

LOQ = 3 µg/kg; LOD = 1 µg/kg.

Reference AH ID 016000en.pdf

Method for determining the residue on flumethrin in bees' honey and wax. Heukamp, U. and Krebber, R. (1993). Report No.RA-654/93 (Method 00339), Bayer AG.

Non-GLP compliant study

This method is a revised version of, and supersedes, Method RA-677/92 (Method no. 00317). The documentation of the raw data in the Appendix to Method RA-677/92 is also applicable to the method described here.

All the reagents and equipment were listed. Samples were separated into honey and wax fractions by heating to 90 °C, allowing the wax to set and scraping off the upper wax layer with a knife.

Honey residue extraction:

Test portions (50 g), were homogenised (2 min) with 100 ml toluene:dichloromethane (DCM):methanol (5:4:1 v/v/v), and the supernatant dried with 25 g sodium sulphate (10 min). The supernatant and 2 × 10 ml DCM salt washings of the precipitate were filtered, evaporated to dryness and resuspended in 7.5 ml ethylacetate:cyclohexane (1:1 v/v). 5 ml of the residue solution was added to a gel permeation chromatography column (GPC: Bio beads SX3, 5 ml/min) and resolved using a ethylacetate:cyclohexane (1:1 v/v) mobile phase. The first 60 ml of GPC eluate was discarded, the next 85 ml of eluate (residue fraction) was collected, evaporated to dryness and the residue resuspended in 1 ml toluene. Solid phase extraction (SPE) on silica gel (10 g; 70-230 mesh, Merck) was achieved by sequentially applying 50 ml n-hexane, the GPC residue, 5 × 2 ml n-hexane rinsings, 150 ml n-hexane:DCM (7:3 v/v), 50 ml n-hexane:DCM (eluate 1), 150 ml n-hexane:DCM (1:1 v/v; eluate 2). The pooled eluates were evaporated to *ca.* 1 ml, transferred to a 10 ml vessel with acetonitrile (ACN) washings, reduced to dryness and resuspended in 0.5 ml ACN. The concentration correction factor in this method for extracts was ×0.015.

Wax residue extraction:

Test portions (10 g), were extracted twice by warming to boiling in 50 ml 2-propanol and 50 ml methanol, cooling (ice bath) and removing the supernatant by filtration. The two filtrates and methanol wax washings were combined and reduced to *ca.* 5 ml by evaporation. The filtrate and 100 ml water were twice extracted with 100 ml ethylacetate:cyclohexane (1:1 v/v) and the organic phases pooled. The organic phases were dried with sodium sulphate (25 g, 15 min), filtered, and evaporated to dryness. The residue was resuspended in 50 ml ACN, washed with 50 ml n-hexane, reduced to dryness and resuspended in 1 ml toluene. Solid phase extraction (SPE) on silica gel (10 g; 70-230 mesh, Merck) was achieved as for honey samples. The residue was resuspended in 1 ml ACN, which corresponded to a correction factor of ×0.10 for concentrations in wax.

Flumethrin residues were determined quantitatively by high-pressure liquid chromatography and UV detection (HPLC-UV) at a wavelength of 266 nm.

The residue, i.e., the content as measured, is calculated from the areas or heights of the peaks obtained with the measurement solution, by reference to those obtained with the external standard.

Method Validation

Specificity: this area was not addressed.

Accuracy: flumethrin was spiked into honey and wax samples (n = 5; 50 g and 10 g respectively) and calibrated against external standards.

Precision: this was tested as indicated in the accuracy section above.

Table 12. The recoveries and CV % of flumethrin determinations in honey and wax

Matrix	Spike (µg/l)	n =	Mean recovery (%)	CV %
Honey	3	5	74	2
	13	5	86.6	1
	85	5	85.9	3
Wax	26	5	59.6	7
	51	5	79.2	13
	102	5	76.2	19

Limit of Detection: these were reported to have been 1 and 20 µg/kg for honey and wax samples respectively.

Limit of Quantification: these were reported to have been 3 and 26 µg/kg for honey and wax samples respectively.

Sensitivity: the linearity of the analytical method was not reported in this study.

Susceptibility to interference: The linearity of the method was demonstrated in the range 0-70 mg/l.

Reference AH ID 013910en.pdf

Analytical method for the determination of Bayvarol (active ingredient flumethrin) in honey and wax. Heukamp, U. (1993). Report No.RA-441/92 (**method 00317**), Bayer AG.

Non-GLP compliant study.

Honey is extracted with a mixture of toluene, dichloromethane and methanol (5:4:1 v/v). After evaporation to dryness the residue is re-dissolved in ethylacetate/cyclohexane (1:1 v/v) and further cleaned by gel-permeation-chromatography. This is followed by a clean-up with silica gel. The quantitative determination is performed by HPLC with UV-detection.

Wax is extracted by boiling with 2-propanol and methanol. After adding of water and cooling down the suspension is filtered and the extract is evaporated to a small volume. After clean-up

by partitioning, the extract is further cleaned with silica gel as in the case of honey. The quantitative determination is performed by HPLC with UV-detection.

Method validation

The method was validated in the laboratory of the author by conducting recovery experiments. Given amounts of standard substance were added to untreated samples of honey and wax. The samples were then analysed according to the method as described above.

Recoveries

Table 13. Honey

Amount added (µg/kg)	Recoveries (%)	Mean (%)	Relative standard deviation (RSD)
3	74, 75, 76, 72, 73	74	0.02
13	85, 87, 86, 88, 87	87	0.01
85	87, 86, 89, 84, 83	86	0.03

Table 14. Wax

Amount added (µg/kg)	Recoveries (%)	Mean (%)	Relative standard deviation (RSD)
26	60, 54, 65, 62, 57	60	0.07
51	78, 90, 65, 75, 89	79	0.13
102	63, 61, 82, 96, 80	76	0.19

Limit of Detection

Control samples of honey and wax were analysed. In the case of honey, there was no peak detected at the retention time of flumethrin, the noise of the baseline was used to determine the limit of detection, using the mean recovery and adding 3 x the standard deviation of the mean.

Two wax samples were used for method validation, there was a small blind value peak found at the retention time of flumethrin corresponding to 8-12 µg/kg of flumethrin.

Therefore, in this case the limit of detection was defined as twice the height of this blind value peak.

Table 15. LOD

Sample	No. of analyses	LOD (µg/kg)
Honey	3	1
Wax	2	20

Limit of Quantification

The LOQ, defined as the lowest concentration detected with good recovery results is 3 µg/kg for honey and 26 µg/kg for wax. No further details were given.

Linearity

The linearity of the detector used for method validation was checked in the range of 85 µg/l to 85 171 µg/l, corresponding to concentrations of:

Honey: 1 µg/kg to 1271 µg/kg in matrix.

Wax: 9 µg/kg to 8517 µg/kg in matrix.

Reference AH ID 0106001en.pdf

Liquid chromatographic method to determine the content of Bayvarol (FCR 1622) in honey and beeswax. Riegner, K. (1986). Report No.RA-197/86, Bayer AG.

Non-GLP compliant study.

Honey is extracted with a mixture of toluene, dichloromethane and methanol (5:4:1). The raw extract is then purified by gel permeation chromatography (GPC) and then over silica gel. The quantitative final determination is performed using high-pressure liquid chromatography (HPLC) with UV detection at 254 nm. The recovery rate from honey is 63 % and the determination limit 2 µg/kg.

Wax is extracted from combs by melting at 90 °C. This wax is dissolved in heated isopropanol and precipitated out again by adding methanol and water. Suctioning and repetition of the extraction procedure are followed by dispersion for further extract purification, using water and a mixture of ethyl acetate/cyclohexane (1:1) and subsequently using acetonitrile and hexane.

As for honey, the final determination is made after final purification over silica gel, using HPLC and UV detection.

The recovery rate from wax combs is 62 % and the determination limit (LOQ) is 25 µg/kg.

All the reagents and equipment were listed. Samples were separated into honey and wax fractions by heating to 90 °C, allowing the upper wax layer to set and scraping off the wax with a knife.

Residue extraction: honey and wax extractions were carried out as in method 654/93.

Method Validation**Recovery (in matrix)**

Flumethrin was dissolved in acetone and added to the honey or wax. To determine the wax yield, the wax was melted once again.

Table 16. Honey

Amount added (µg/kg)	Recovery (%)	Mean (%)
3.1	61, 63	62
3.8	54, 74	64
4.4	58, 62, 70	63

The mean recovery rate from honey is 63 % of the added quantity of flumethrin with a confidence interval of ± 6.3 % and 95 % statistical certainty.

Table 17. Wax

Amount added (µg/kg)	Recovery (%)	Mean (%)
25	58, 66	62
50	53, 69	61
100	58, 63, 69	63

In the concentration range tested, the mean recovery rate from wax is 62 % of the added flumethrin, with a confidence interval of ± 5.7 % and 95 % statistical certainty.

Limit of Quantification

Honey: 2 µg/kg

Wax: 25 µg/kg (15 µg/kg when taken up in 1 ml acetonitrile)

Reference *AH ID 041150en.pdf*

Analytical method for the determination of flumethrin in bees honey and wax by LC-MS/MS. Report number: MR-15/101. Study number P 603 155025 (Krebber, Hoffend & Anjard, 2015). Method 01462.

LOQ: Honey: 3 µg/kg; Wax: 25 µg/kg

Honey is extracted by shaking with water for 10 minutes, and then acetonitrile is added and mixed. The extract is cleaned-up using a C18 SPE cartridge, and the analyte eluted with acetonitrile. The extract is evaporated to dryness, re-dissolved in acetonitrile and, if necessary, filtered prior to determination by LC-MS/MS.

Wax is extracted with ethanol by heating to 70 °C for 15 minutes, and then shaking for 30 minutes. The extract is placed in dry ice for 15 minutes (or in a freezer at -18 °C overnight), and then centrifuged. A portion of the supernatant is evaporated, redissolved in isohexane and cleaned-up by liquid-liquid partition with acetonitrile (saturated with isohexane). The acetonitrile extract is evaporated to dryness, redissolved in acetonitrile and filtered prior to determination by LC-MS/MS.

LC-MS/MS is performed in positive ionisation mode. Two mass transitions can be monitored; m/z 527 to 267 (quantification transition) and m/z 527 to 239 (confirmation transition).

The method has been validated with an acceptable range of recoveries (70 %-120 %) and relative standard deviations (RSD = < 20 %).

Table 18. Accuracy and Precision Data

Matrix	Analyte	Fortification Level (µg/kg)	Range (%)	Recovery	n	Mean (%)	RSD (%)
Honey (quantification m/z 527 → m/z 267)	Flumethrin	3	78-97		6	85	8.6
		6	78-90		6	86	4.9
		30	77-90		6	83	5.3
		Overall	77-97		18	85	6.4
Honey (confirmation m/z 527 → m/z 239)	Flumethrin	3	80-98		6	88	8.8
		6	76-94		6	86	6.7
		30	79-92		6	84	5.8
		Overall	76-98		18	86	7.1
Wax (quantification m/z 527 → m/z 267)	Flumethrin	25	87-103		6	96	6.7
		50	98-104		6	101	2.1
		250	87-108		6	101	7.4
		Overall	87-108		18	99	6.2
Wax (confirmation m/z 527 → m/z 239)	Flumethrin	25	66-96		6	83	19
		50	87-113		6	99	9.4
		250	85-103		6	96	7.0
		Overall	66-113		18	92	13

Reference ID 41149

Determination of flumethrin in honey and wax. Report No.: MR-15/183. Study Number: P673155038 (Krebber & Hoffend, 2015)

The honey and wax samples were analysed for concentrations of flumethrin by LC-MS/MS. Flumethrin was extracted from honey by shaking with water and acetonitrile. The extract was cleaned-up by solid phase extraction. Wax was extracted with ethanol at 70 °C and half of the extract was used for further clean-up. After elimination of the wax by freezing, the solvent was evaporated. The remainder is dissolved in iso-hexane and extracted by partition with acetonitrile. The extract was evaporated to dryness and dissolved in acetonitrile.

The quantitative determination was performed by HPLC with a tandem mass spectrometric detector. The limit of quantitation was 3 µg/kg in honey and 25 µg/kg in wax.

The measured concentration was calculated by comparison of the analyte response to a standard calibration curve obtained from matrix-matched standards.

Method validation

The method was validated for honey and wax before and during the analyses by concurrent recoveries.

Table 19. Recovery rates for flumethrin in honey

Sample material	Fortification level (µg/kg)	Recovery (%)						Mean value (%)	RSD (%)
Honey	3	80	82	83	97	91	78	85	8.6
	6	90	87	86	78	88	88	86	4.9
	30	85	90	81	77	82	81	83	5.3
	Mean	n = 18						85	6.4

RSD = Relative standard deviation

Table 20. Recovery rates for flumethrin in wax

Sample material	Fortification level (µg/kg)	Recovery (%)						Mean value (%)	RSD (%)
Wax	25	91	102	98	87	93	103	96	6.7
	50	102	100	103	101	104	98	101	2.1
	250	108	87	103	104	106	100	101	7.4
	Mean	n = 18						99	6.2

RSD = Relative standard deviation

Specificity:

The high selectivity of the method resulted from the HPLC separation in combination with MS/MS detection.

Linearity:

The correlation between the injected amount of substance and the detector response was linear (1/x weighted) for matrix-matched standard solutions ranging from 0.5 to 150 µg/l (corresponding to 1 to 300 µg/kg in the sample) for honey and between 1 and 50 µg/l for wax (corresponding to 10 to 500 µg/kg in the sample). The correlation coefficients were ≥ 0.996 for both matrices.

Accuracy and precision:

For precision, repeatability and within-laboratory reproducibility data were provided. The coefficient of variation (CV (= RSD)) was used as the measure.

Table 21. Recovery rates for flumethrin in honey for the quantitation ion (m/z 527 → m/z 267)

Sample material	Fortification level (µg/kg)	Recovery (%)						Mean value (%)	Accuracy (%)	CV (%)
Honey	3	80	82	83	97	91	78	85	-15	8.6
	6	90	87	86	78	88	88	86	-14	4.9
	30	85	90	81	77	82	81	83	-17	5.3
	Mean	n = 18						85	-15	6.4

Table 22. Recovery rates for flumethrin in wax for the quantitation ion (m/z 527 → m/z 267)

Sample material	Fortification level (µg/kg)	Recovery (%)						Mean value (%)	Accuracy (%)	CV (%)
Wax	25	91	102	98	87	93	103	96	-7	6.7
	50	10	100	103	101	104	98	101	1	2.1
	250	10	87	103	104	106	100	101	-1	7.4
	Mean	n = 18						99	-1	6.2

Limit of Quantitation (LOQ):

The limit of quantitation is 3 µg/kg in honey and 25 µg/kg in wax, based on the lowest fortification levels tested.

Limit of Detection (LOD):

The detection limits (LOD) were calculated based on a statistical approach for each sample material. The LODs were calculated to be 0.74 µg/kg for honey and 10 µg/kg for wax.

Table 23. Calculated LOD for honey

Sample material	Fortification level (µg/kg)	Residue detected (µg/kg)
Honey	0	0
	0	0
	0	0
	Replicates	3
	Average	0
	3	2.40
	3	2.46
	3	2.49
	3	2.91
	3	2.73
	3	2.56
	Replicates	6

Average	2.56
Standard Deviation (SD)	0.22
Calculated LOD	0.74
Calculated LOD = (3.365 x SD + average residue in untreated controls), where 3.365 is Student t-factor for n = 6 replicates.	

Table 24. Calculated LOD for wax

Sample material	Fortification level (µg/kg)	Residue detected (µg/kg)
Wax	0	0
	0	0
	0	0
	Replicates	3
	Average	0
	25	22.5
	25	25.5
	25	24.5
	25	17.5
	25	23.3
	25	25.8
	Replicates	6
	Average	23.2
	Standard Deviation (SD)	3.05
Calculated LOD	10.3	
Calculated LOD = (3.365 × SD + average residue in untreated controls), where 3.365 is Student t-factor for n = 6 replicates.		

Matrix Effects:

The MS/MS detection of flumethrin is affected by the matrix. For honey, peak area for standard in solvent was about 84 % of the peak area obtained for matrix-matched standards. For wax the peak area decreased to about 20 % compared to the peak area found for standards in solvent.

Stability of residues**Reference AH ID 042560en.pdf**

Study of Acaricide Stability in Honey. Characterization of Amitraz Degradation Products in Honey and Beeswax (Korta *et.al.*, 2001).

Only the information relevant to the stability of flumethrin in honey has been reported below. No data on beeswax were included.

Honey:

Honey samples were spiked with flumethrin at 10 mg/kg. This spiked honey was kept in the closed glass container at room temperature (20-25 °C) for 9 months, away from exposure to direct sunlight. Samples were analysed (using an HPLC method) at day 0, 3 months and 9 months. There was no appreciable decline in concentration of flumethrin in the honey (results were 9.8, 10, And 9.9 mg/kg, respectively).

From Reference ID 41150:

Determination of flumethrin in honey and wax. Report No.: MR-15/183. Study Number: P673155038 (Krebber & Hoffend, 2015)

Stability:

Solutions of the reference substance flumethrin in solvent are stable for at least 3 months when stored in a refrigerator ≤ 7 °C.

Analytical solutions of honey and wax samples were stable for at least 5 days when stored in a refrigerator ≤ 7 °C.

The stability of flumethrin in stored samples was demonstrated in honey and wax samples for a period of one month at ambient temperature and ≤ -18 °C.

Appraisal

No radiolabeled residue depletion study has been conducted or provided. These data are not required for substances in honey, as there is no known metabolism of xenobiotics in honeybees and in this case there is also no degradation in honey.

Between-comb strips (honey):

Six non-GLP-compliant residues depletion studies of flumethrin in honey were conducted in various locations in Germany and the UK in the 1980s and 1990s. All these studies used LDPE between-comb strips impregnated with 3.6 mg flumethrin per strip.

In one study, 24 beehives were treated with 4 strips each, corresponding to a total dose of 14.4 mg flumethrin, for a duration of 6 months, over the winter period. One sample was analysed, no residues were detected (LOQ = 3 µg/kg; LOD = 1 µg/kg). Additionally, 12 beehives were treated for 6 weeks with 4 strips per hive, from the beginning of September to the middle of October, over 2 years. Four samples were analysed, no residues were detected.

In the second study, an unreported number of beehives were treated with 4 strips per brood chamber for six weeks, after the honey harvest. One sample was taken, during the spring honey flow. No residues were detected. (LOQ = 3 µg/kg; LOD = 1 µg/kg).

In the third study, six beehives were treated for 6 weeks, from September to October, with 4 strips per frame. Six samples were taken in June the next year and combined in pairs to form three samples for analysis. No residues were detected above the LOQ of 2 µg/kg.

In the fourth study, six beehives were treated for 5 months from October to March with 4 strips per frame. Six samples of freshly capped honeycomb were taken in June of the following year, after the early nectar flow. No residues were detected above the LOQ of 2 µg/kg.

In the fifth study, seven beehives were treated from early March to mid-April with 4 strips per frame. At the end of the treatment period, these combs were labelled and suspended in the honey chamber until the last brood had hatched and fresh honey had been inserted and capped. No residues were detected above the LOQ of 2 µg/kg.

In the final study of between-comb strips, four beehives were treated for 4 months from May to September, during the honey flow period. The entire honey harvest of each treated hive was centrifuged separately in the last week of August. No residues were detected above the LOQ of 2 µg/kg.

These studies demonstrate that even when used contrary to Good Beekeeping Practice (in doses higher than recommended, or over extended periods of time), or when used during honey flow, no residues of flumethrin were detected in honey. It should be noted, however, that these studies were not conducted to current GLP standards, did not have comprehensive study reports, used slightly different analytical methods (based on HPLC-UV), and had inconsistencies in reporting, that limit the usefulness of the results.

Between-comb strips (beeswax):

Five non-GLP-compliant residues depletion studies of flumethrin in beeswax were conducted in various locations in Germany and Switzerland. All studies used LDPE strips impregnated with 3.6 mg flumethrin per strip. The samples in all studies were analysed using HPLC-UV.

In the first study, 15 beehives were treated as per product instructions (4 strips per brood chamber for six weeks, after the honey harvest) in three Swiss cantons. Thirteen samples of beeswax were analysed for flumethrin and residues were found in the range < 26 µg/kg to 176 µg/kg. Mean residues were around 50 µg/kg. The LOQ for the analytical method used was 26 µg/kg.

In the second study, an unreported number of beehives were treated with ten times the recommended number of strips (40 strips per hive). The analysis of four samples was reported and the results were in the range 70 – 146 µg/kg; mean = 106 µg/kg. The LOQ of the analytical method used was reported as 26 µg/kg.

In the third study, two beehives were treated with 4 strips per chamber for 6 weeks, just before the start of honey flow. Two samples per frame were analysed. Residues in the range < 15 to 40 µg/kg were reported. The LOQ of the analytical method used was 15 µg/kg.

In the fourth study, six beehives were treated with 4 strips per brood chamber for 6 weeks, from September to October, after the honey harvest. The following year, samples were taken in June, after the early honey flow. One honeycomb was taken from each hive and combined with one from another beehive to form three samples, which were then analysed for flumethrin. The results of the analyses showed residues in the range of < 20 - 50 µg/kg. The LOQ of the analytical method used was 20 µg/kg.

In the final study, four beehives were treated with 4 strips per brood chamber for six months, during the honey flow period. The strips were inserted in May and removed in September. Two combs from each hive that had been in the brood chamber were moved to the honey chamber in mid-August until the brood hatched. These combs were sampled and analysed. The results were in the range 30 - 130 µg/kg. The LOQ of the analytical method used was reported as 20 µg/kg.

These studies demonstrate that flumethrin has more affinity for beeswax than the honey, which is to be expected considering the lipophilic nature of flumethrin ($\text{Log } P_{\text{ow}} = 6.2$). Again, it should be noted that these studies were not conducted to current GLP standards, did not have comprehensive study reports, used slightly different analytical methods (based on HPLC-UV), and had inconsistencies in reporting that limit the usefulness of the results.

Beehive gates (honey and wax):

The sponsor provided one GLP-compliant field study conducted in 2015 in beehives, using beehive gate strips. Honeycombs were obtained from different test sites in Germany (4 apiaries), Hungary (2 apiaries), Spain (2 apiaries) and The Netherlands (3 apiaries) after application of flumethrin bee-hive gates (275 mg per gate). Both honey and beeswax were sampled and analysed.

Thirty-two honeycomb samples from four apiaries in Germany, 14 samples from two apiaries in Hungary, 12 samples from two apiaries in Spain and 18 samples from three apiaries in The Netherlands were analysed.

The maximum recommended application time at the hive entrance of 4 months was covered by data from Germany (122 or 120 days) and the Netherlands (119 days). In the other regions, application time was 92 days (Spain) and 102 days (Hungary).

For residue analysis honey from all hives of one apiary were pooled, as was the wax. Equal amounts of the previously purified honey samples were combined and homogenised by stirring at 40 °C. Wax samples were melted at around 63 °C, homogenised and purified from contamination (e.g., hive detritus) by skimming off or passing it again through a sieve.

The honey and wax samples were analysed for flumethrin using a validated LC-MS/MS method.

Residue concentrations in honey were below the limit of quantification of 3 µg/kg. Concentrations in beeswax were above the LOQ of 25 µg/kg in three out of 11 samples. The highest residue in wax was 119 µg/kg.

Analytical methods

Several versions of the HPLC-UV method used to analyse the samples generated from the studies conducted using the between-comb strips in the 1980s-90s to determine residues of flumethrin in honey and wax were provided, although none of them were well-reported or validated according to current standards.

The sponsor also provided the details of an LC-MS/MS method that was used to determine residues of flumethrin in honey and wax after use of the beehive gates. This study was conducted in a GLP-accredited laboratory and the method validated according to international requirements. Flumethrin was extracted from honey and wax. The quantitative analysis was performed using LC-MS/MS. The limit of quantitation was 3 µg/kg in honey and 25 µg/kg in wax.

Dietary Exposure Assessment

Exposure to flumethrin residues may occur through its use as a pesticide as well as a veterinary drug.

Dietary exposure from pesticide residues (IEDI)

When used as a pesticide, the exposure of flumethrin was found to be below the upper bound of the ADI of 0.004 mg/kg bw (JMPR, 1996). The sources of exposure considered were cattle meat and milk.

Dietary exposure from veterinary drug residues (GECDE)

JECFA has proposed an ADI of 0-0.004 mg/kg bw and an ARfD of 0.005 mg/kg bw. The toxicological profile of flumethrin requires exposure estimates for children at the highest reliable percentile of food consumption based on consumers only.

When used as a veterinary drug, chronic and acute dietary exposure in the general population and in children was estimated based on the potential occurrence of residues in honey and beeswax. It was assumed that in all cases honey is consumed with beeswax (as occurs in comb honey and raw honey) and the honey to wax ratio is 9:1 (JECFA 2008). This is a conservative assumption, as most commercial honey is likely to contain much less beeswax. Other honeybee products, such as propolis, royal jelly and pollen, were not included in the exposure assessment.

The GECDE for the general population is 0.008 µg/kg bw per day (Table 25), which represents 0.2 % of the upper bound of the ADI of 0.004 mg/kg bw set by JECFA during this meeting. The GECDE for children is 0.006 µg/kg bw per day, which represents 0.2 % of the upper bound of the ADI.

In addition to the accepted GECDE methodology, further calculations were carried out. Instead of using the highest mean and the highest 97.5th percentile consumption across surveys, the calculations were carried out using the mean and the highest reliable percentile for each individual national survey from available data sets (CIFOcOss). The mean of all the GECDE calculations across surveys were reported. The mean of 21 estimates was 0.002 µg/kg bw per day (0.05 % of the upper bound of the ADI), with a range of <0.0001-0.008 (<0.003-0.2 % of the upper bound of the ADI). For children, chronic dietary exposure could be estimated from 43 individual studies. The mean of these 43 studies was 0.001 µg/kg bw per day (0.03% of the upper bound of the ADI), with a range of <0.0001-0.006 (<0.003-0.2 % of the upper bound of the ADI).

Dietary exposure from veterinary drug residues (GEADE)

The GEADE for the general population is 0.1 µg/kg bw per day (Table 26), based on consumption of wax contained in honey, which represents 2.2 % of the ARfD of 0.005 mg/kg bw per day. The GEADE for children is also 0.1 µg/kg bw per day, based on consumption of wax contained in honey, which represents 2.2 % of the ARfD.

Combined chronic dietary exposure from pesticide and veterinary drug residues (Extended GECDE)

Modified methods based on the GECDE were used to estimate combined chronic dietary exposure. The usual GECDE approach was extended to include additional commodities that were assessed for the compound by JMPR (Extended GECDE). It should be noted that this new exposure assessment methodology is still being piloted. It should further be noted that the median residues used as inputs were extracted from JMPR publications and have not been validated for this assessment.

Combined chronic dietary exposure from veterinary drug and pesticide residues was considered for the general population and in children, based on the potential occurrence of residues in honey, beeswax, cattle meat and cattle milk. Assumptions for honey and beeswax were the same as for the GECDE.

The Extended GECDE for the general population is 0.179 µg/kg bw per day (Table 27), which represents 5 % of the upper bound of the ADI of 0.004 mg/kg bw set by JECFA during this meeting. The Extended GECDE for children is 1.008 µg/kg bw per day, which represents 25 % of the upper bound of the ADI. For both populations cattle milk was the major contributor to chronic dietary exposure.

Table 25. Estimated chronic dietary exposure to flumethrin (GECDE) occurring in honey and associated beeswax

Category	Type	Median concentrat ion ¹ (µg/kg)	Mean consumption ² (whole population, g/kg bw/day)	Highest percentile consumption ³ (consumers only, g/kg bw) /[percentile used]	reliable consumption ³ g/kg	MR:TR ratio	Exposure (µg/kg bw/day)	mean	97.5th	GECDE ⁴ µg/kg bw/day	ADI %
General Population											
Honey	Honey	2	0.036	1.9 [97.5]		1	0.00007		0.004		
Honey	Beeswax ⁵	40	0.004	0.2 [97.5]		1	0.00016		0.008		
TOTAL							0.00007		0.008		0.2
Children											
Honey	Honey	2	0.136	1.3 [97.5]		1	0.00027		0.003		
Honey	Beeswax ⁵	40	0.015	0.1 [97.5]		1	0.00061		0.006		
TOTAL							0.00027		0.006		0.2

¹Median concentration at the end of treatment

²highest mean consumption figures based on whole population considered from the available dataset

³highest food consumption figures based on consumers only considered from the available dataset

⁴GECDE is the sum of the highest exposure at the highest reliable percentile of consumption for a food and the mean dietary exposures of the other foods

⁵Beeswax in honey only, calculated by assuming beeswax consumption is 1/9 of honey consumption

Table 26. Estimated acute dietary exposure to flumethrin (GEADE) occurring in honey and associated beeswax

Category	Type	95 th centile concentration ¹ (mg/kg)	Acute Consumption ² (g/kg bw)	MR:TR ratio	GEADE ³ mg/kg bw/day	ARfD %
General Population						
Honey	Honey	0.002	5.5	1	0.01	0.2
Honey	Beeswax ⁴	0.176	0.6	1	0.11	2.2
Children						
Honey	Honey	0.002	5.5	1	0.01	0.2
Honey	Beeswax ⁴	0.176	0.6	1	0.11	2.2

¹concentration at the end of treatment²highest food consumption figures based on the 97.5th percentile consumption from available data set³GEADE is the product of the 97.5th level of consumption multiplied with the highest residue⁴Beeswax in honey only, calculated by assuming beeswax consumption is 1/9 of honey consumption

Table 27. Estimated chronic dietary exposure to flumethrin (Extended GECDE) occurring in honey, associated beeswax, cattle meat and cattle milk

Category	Type	Median concentration ¹ (µg/kg)	Mean consumption ² (whole population, g/kg bw/day)	Highest reliable percentile consumption ³ (consumers only, g/kg bw) / [percentile used]	MR:TR ratio	Exposure		GECDE ⁴	
						mean (µg/kg bw/day)	97.5th	µg/kg bw/day	ADI %
General Population									
Honey	Honey	2	0.036	1.9 [97.5]	1	0.00007	0.004		
Honey	Beeswax ⁵	40	0.004	0.2 [97.5]	1	0.00016	0.008		
Mammalian meat	Beef and other bovines	10	0.959	4.4 [97.5]	1	0.00959	0.044		
Milk	Cattle Milk	10	4.4	16.9 [97.5]	1	0.04400	0.169		
TOTAL						0.00982	0.169	0.179	5
Children									
Honey	Honey	2	0.136	1.3 [97.5]	1	0.00027	0.003		
Honey	Beeswax ⁵	40	0.015	0.1 [97.5]	1	0.00061	0.006		
Mammalian meat	Beef and other bovines	10	8.349	1.9 [97.5]	1	0.01940	0.084		
Milk	Cattle Milk	10	98.74	42.6 [97.5]	1	0.42590	0.987		
TOTAL						0.02028	0.987	1.008	25

¹Median concentration at the end of treatment
²highest mean consumption figures based on whole population considered from the available dataset
³highest food consumption figures based on consumers only considered from the available dataset
⁴GECDE is the sum of the highest exposure at the highest reliable percentile of consumption for a food and the mean dietary exposures of the other foods
⁵Beeswax in honey only, calculated by assuming beeswax consumption is 1/9 of honey consumption

Risk management considerations

It is noted that beeswax may be present in food and originate from a variety of sources; because of this, and the fact that flumethrin accumulates in the wax, it is considered that risk management measures around the use of beeswax that may contain residues of flumethrin could be applied.

An example is where bee keepers reuse the wax combs season after season. This is common practice, as it takes a lot of energy for the bees to make the wax combs, so in order to maximise honey production, the combs are reused. It might therefore be prudent to advise beekeepers to limit reuse of their combs if they are using products that contain flumethrin on their hives. Another measure might be to recommend not using the same active ingredient in subsequent years, so to rotate the available products year by year. This may also reduce the likelihood of resistance to flumethrin of the target parasites.

No data on residues of flumethrin have been evaluated with regard to other products derived from beehives (e.g., propolis, royal jelly, etc.), therefore, no risk management proposals can be made by the Committee for these commodities.

Maximum Residue Limits

In recommending an MRL for flumethrin in honey, the Committee considered the following factors:

- An ADI for flumethrin of 0–0.004 mg/kg bw was established by the Committee.
- An ARfD of 0.005 mg/kg bw was established by the Committee.
- In view of the toxicological profile of flumethrin, specific exposure scenarios are required to address exposure of pregnant women, infants and young children and high percentile adult consumers.
- Flumethrin is used both as a pesticide and a veterinary drug.
- Flumethrin is authorised for use in beehives in several countries. The maximum recommended dose is 275 mg x 2, administered via PVC beehive gates, or 3.6 mg × 4 administered via between-comb LDPE strips.
- The withdrawal period for all products is zero days.
- Flumethrin is the marker residue in honey.
- The ratio of the concentration of marker residue to the concentration of total residue of 1.0 was calculated in honey and beeswax.
- A validated analytical method for the determination of flumethrin in honey and beeswax is available and may be used for monitoring purposes.

Although there were no quantifiable residues found in honey after treatment with the flumethrin products evaluated, the Committee, in response to the request of the 23rd session of CCRVDF, could set an MRL for honey at twice the LOQ of the analytical method used in the residues studies. Because the most reliable method was the more recent LC-MS/MS method, which had an LOQ of 3 µg/kg, the Committee recommended an MRL of 6 µg/kg.

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Halquinol

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Identity

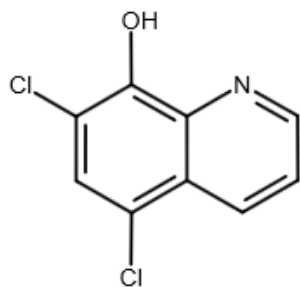
International Non-proprietary Names (INN): Halquinol

Synonyms: Halquinol BP 80, Chloroquinol, CHQ

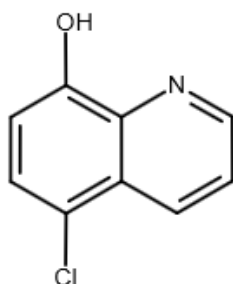
IUPAC name: 5,7-dichloro-8-hydroxy quinoline; 5-chloro-8-hydroxy quinoline; 7-chloro-8-hydroxy quinoline

Chemical abstract service N°: 8067-69-4; 130-16-5; 876-86-8

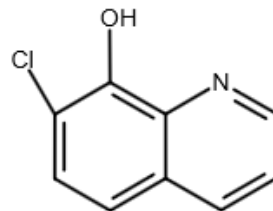
Structural formula: Structures of 5,7-dichloro-8-hydroxy quinoline (5,7-DCL), 5-chloro-8-hydroxy quinoline (5-CL) and 7-chloro-8-hydroxy quinoline (7-CL), are shown below:



5,7-DCL



5-CL



7-CL

Molecular formula: The molecular formulas of the three constituent molecules of halquinol are:

5,7-dichloro-8-hydroxy quinoline: $C_9H_5Cl_2NO$

5-chloro-8-hydroxy quinoline and 7-chloro-8-hydroxy quinoline: C_9H_6ClNO

Molecular weight: The molecular weights of the three constituent molecules of halquinol are:

5,7-dichloro-8-hydroxy quinoline: 214.1

5-chloro-8-hydroxy quinoline and 7-chloro-8-hydroxy quinoline: 179.6

Other information on identity and properties

Pure active ingredient: Halquinol

Appearance: Cream to pale green, fine powder

Melting point: 145-165 °C

Solubility: Insoluble in water; soluble in 250 parts ethanol (96 %), in 130 parts ether and in 50 parts chloroform

Residues in food and their evaluation

Conditions of use

Halquinol (trade name Quixalud®) is an antimicrobial used as a feed additive for poultry and as a growth promotant in pigs. Halquinol also is used in swine for the control, treatment and prevention of scours caused or complicated by *E. coli* and *Salmonella spp.* Halquinol for swine is approved in multiple Asian and Latin American countries and feed concentrates range from 12-60 % halquinol, with varying dose regimens and withdrawal periods (Table 1).

Table 1. Halquinol Product Approvals and Withdrawal Periods

Country	Dose (ppm)	Indication	Withdrawal Period
Quixalud 60 %			
Philippines	100-200	Treatment and prevention of chronic swine scour and diarrhea. Treatment of mold infection.	Standard of 5-7 days as required by local authority
Taiwan	500-600	Treatment and prevention of chronic swine scour and diarrhea. Treatment of mold infection.	2 days
Thailand	120	Prevention and treatment of scours in pigs associated with <i>Salmonella</i> and <i>E. coli</i> .	7 days
Brazil	60	Prevention and treatment of scours in pigs associated with <i>Salmonella spp.</i> and <i>E. coli</i> .	4 days
Argentina, Bolivia, Paraguay	100-200	Treatment of diarrhea, specifically due to <i>E. coli</i> and <i>Salmonella spp.</i> , but also diarrhea caused by diet, fungi or protozoa. Antidiarrheal agent with active improvement of performance.	No withdrawal period
Colombia	60-120	Growth promoter and feed efficiency improver.	No withdrawal period
Quixalud 60 NF			
Colombia, Ecuador, Peru	60-120	Control and treatment of diarrhea due to or complicated by <i>E. coli</i> and <i>Salmonella spp.</i> , but also unspecific	4 days

		diarrhea caused by fungi (<i>Candida albicans</i> , <i>Aspergillus spp.</i>) and protozoa (<i>Entamoeba spp.</i> , <i>Trichomonas spp.</i>)	
Quixalud 12 %			
Peru, Panama, Ecuador, Venezuela, El Salvador, Nicaragua, Honduras, Costa Rica, Colombia, Dominican Republic, Guatemala	60-120	Growth promoter and feed efficiency	No withdrawal period
Peru, Panama, Ecuador, Venezuela, El Salvador, Nicaragua, Honduras, Costa Rica, Colombia, Dominican Republic, Guatemala	600	Treatment of chronic diarrhea	No withdrawal period
Other Product Names			
India	Feed Supplement	Feed supplement	No withdrawal period
Vietnam	120	Prevention and treatment of scours in pigs associated with <i>Salmonella</i> and <i>E. coli</i> .	7 days

The sponsor reported that halquinol has been approved for over a decade in Thailand, Vietnam, Indonesia and Taiwan and 6 years in the Philippines. During the initial registration of halquinol in these countries, human food safety data (including ADME, residue depletion, etc.) were not required. Withdrawal time was set by default by the respective authorities.

Australia completed a special review of halquinol in 1996 but did not publish a final report, as the registrant had withdrawn all products before the completion of the review. The Australian Pesticides and Veterinary Medicines Authority (APVMA) concluded that there were insufficient toxicological data to support the continued registration of halquinol or its associated MRLs. The APVMA recommended cancelling the active constituent approval for halquinol, cancelling the MRLs for halquinol and cancelling the registrations of all products containing halquinol, effective March 31, 1996. There are no veterinary products containing halquinol currently registered in Australia for the treatment of food-producing animals.

Dosage

Halquinol is composed of a mixture of chlorinated products of quinolin-8-ol. Chlorinating quinolin-8-ol yields a mixture, generically called halquinol, which contains 5,7-dichloroquinolin-8-ol (5,7-DCL; 57-74 % w/w), 5-chloroquinolin-8-ol (5-CL; 23-40 % w/w) and 7-chloroquinolin-8-ol (7-CL; 0-4 % w/w). The product, available under the trade name Quixalud, is composed of chlorohydroxyquinoline (halquinol; 60 % w/v), silicon dioxide (1.2 % w/v) and chalk (calcium carbonate; to 100 % w/v). Halquinol is administered to swine orally in the feed at a dose inclusion rate varying from 60 to 600 mg halquinol/kg feed (ppm), for up to 10 consecutive days. Based on a typical daily feed intake of approximately 4 % body weight/day for pigs, this results in a dose of approximately 2.4 to 24 mg halquinol/kg bw.

Pharmacokinetics and metabolism

Test material used in all toxicokinetic/pharmacokinetic studies

The toxicokinetic data in laboratory animals (rats, dogs, and minipigs) were derived from studies conducted using the same batch of Halquinol BP 80 (HLA 4067).

Purity: 98.82 %, comprised of 5-chloroquinol-8-ol (5-CL; 26.29 %) and 5,7-dichloroquinol-8-ol (5,7-DCL; 72.53 %). The amount of 7-chloro-8-hydroxy quinoline (7-CL) in this batch was not assessed, but could not account for more than 1.18 % of the total halquinol. Because plasma and/or tissue concentrations of 7-CL were expected to be negligible relative to the other halquinol components and their metabolites, and because 7-CL represents only 0-4 % of the approved formulation, this component was not analysed in any pharmacokinetic or residue depletion study.

Pharmacokinetics in laboratory animals

Studies examining the pharmacokinetics of halquinol in laboratory animals were conducted as part of the toxicology program. Halquinol was administered *via* the oral route, though the doses used were not in the therapeutic dose range as per the label indications for swine. Overall, the pharmacokinetics of halquinol in laboratory animals is similar to swine in that parent halquinol is absorbed quickly (t_{\max} of 1 – 6 h) and rapidly converted to conjugate forms (glucuronides and sulfates).

Rats

As part of a GLP-compliant 13-week toxicity study (Bentz, 2015), three groups of 8 Sprague-Dawley rats (4 male and 4 female)/group each received the test item, Halquinol BP 80 by daily oral gavage. Halquinol was administered at doses of 50, 150 and 450 mg/kg/day. Doses were administered as a suspension in the vehicle [methylcellulose at 0.5 % (w/w) in drinking water], with a constant gavage volume of 5 mL/kg/day.

Blood samples were collected on Day 1 at 0, 1, 3, 6 and 24 h after gavage; and at the end of the treatment period (Day 91) at 0 and 1 hour after gavage. The concentrations of parent halquinol (5-CL and 5,7-DCL) and glucuronided and sulfated metabolites were determined in plasma

using a validated LC-MS/MS method (Decorde, 2016). Noncompartmental kinetic analysis was performed to derive the PK parameters.

Mean pharmacokinetic parameters after halquinol administration in rats is presented in Table 2. Following a single oral administration of halquinol (Day 1), 5-chloroquinolin-8-ol (5-CL) was not quantifiable in plasma samples in any dose group. After repeated oral administration (13 weeks), 5-CL was still not quantifiable in plasma in all animals for dose-levels 50 and 150 mg/kg/day, but was quantifiable in two males and two females at 450 mg/kg/day (1 h post-dose). However, glucuronided and sulfated metabolites of 5-CL (5-CLG, 5-CLS) were rapidly produced, with time to peak concentrations (t_{\max}) observed at the first sample collection (1 hour post-dose) for all rats in the 50 and 150 mg/kg/day dose groups. At the highest dose (450 mg/kg/day), maximum concentrations of 5-CL metabolites were produced between 1-3 h post-dose. After initial halquinol dosing, the mean peak concentrations (C_{\max}) of 5-CLG were 2913, 4304, and 11203 ng/mL for the 50, 150, and 450 mg/kg/day dose groups, respectively. Peak concentrations of 5-CLS were approximately 28 – 40 % of the peak 5-CLG values, at 818, 1616, and 4494 ng/mL for the respective dose groups.

5,7-dichloro-8-hydroxyquinoline (5,7-DCL) and its glucuronide (5,7-DCLG) and sulfate (5,7-DCLS) metabolites were quantifiable in plasma for all dose groups. The t_{\max} for 5,7-DCL and its metabolites increased with increasing dose. For 5,7-DCL and its metabolites, the t_{\max} was observed at the first sampling time (1 hr) for all rats in the 50 mg/kg/day group. In the 150 mg/kg/day dose group, maximum concentrations were observed between 1 – 3 h post-dose, and in the 450 mg/kg/day group between 1–6 h post-dose. After initial halquinol dosing, the mean peak concentrations (C_{\max}) of 5,7-DCL were 245, 800, and 1710 ng/mL for the 50, 150, and 450 mg/kg/day dose groups. Mean C_{\max} values for the metabolites were approximately 5-10x higher than parent: 2607, 4187, and 9013 ng/mL for 5,7-DCLG, and 3009, 4849, and 11552 ng/mL for 5,7-DCLS at the respective dose groups. Unlike 5-CL metabolites, where the peak concentrations of the glucuronide form exceeded the sulfated form, the peak concentrations of 5,7-DCLS exceeded 5,7-DCLG by ~ 23 %.

For all halquinol components, plasma concentrations depleted rapidly and were typically only detectable at 1, 3, and 6 h after the first dose. The exception was 5,7-DCLG, which was detectable at 24-h post-dose in all 8 rats.

After chronic halquinol administration (13 weeks), 5-CLG peak concentrations (C_{\max}) were comparable to those observed after a single dose. Conversely, the peak concentration of 5-CLS after 13 weeks increased dramatically at all doses (3.1x, 3.1x, and 2.2x the initial C_{\max} , for the 50, 150, and 450 mg/kg/day dose groups respectively), suggesting an accumulation of the sulfate form. Similar findings were observed for 5,7-DCL. Peak concentrations were comparable between day 1 and week 13 samples for both 5,7-DCL and its glucuronide metabolite. However, the C_{\max} of 5,7-DCLS was 2.2x, 1.6x, and 1.1x the C_{\max} observed on Day 1 for the respective dose groups.

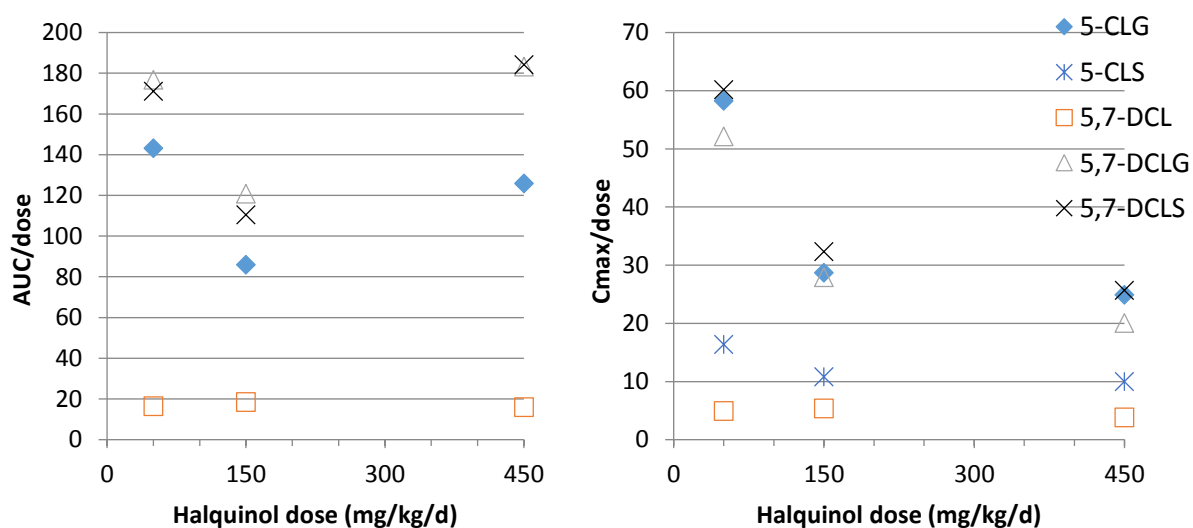
Table 2. Summary of mean pharmacokinetic parameters after halquinol oral gavage in rats (Bentz, 2015)

Dose (mg/kg/day)	Period	Parameter	Unit	5-CL	5-CLG	5-CLS	5,7-DCL	5,7-DCLG	5,7-DCLS
50	Day 1	t_{max}	h	ND	1.0	1.0	1.0	1.0	1.0
		C_{max}	ng/mL	ND	2913	818	245	2607	3009
		AUC_{0-t}	ng*h/mL	ND	7158	ND	817	8837	8559
	Week 13	C_{max}	ng/mL	ND	2794	2521	96	2878	6502
		t_{max}	h	ND	1.2	1.2	1.2	1.6	1.2
		C_{max}	ng/mL	ND	4304	1616	800	4187	4849
150	Day 1	AUC_{0-t}	ng*h/mL	ND	12879	ND	2770	18128	16572
		C_{max}	ng/mL	ND	4614	5055	518	3933	7889
		t_{max}	h	ND	1.6	1.2	2.6	3.0	2.5
	Week 13	C_{max}	ng/mL	ND	11203	4494	1710	9013	11552
		AUC_{0-t}	ng*h/mL	ND	56630	13647	7175	82465	82872
		C_{max}	ng/mL	13.1	11787	9757	1108	6993	12247

ND = not determined

In general, no significant gender difference was observed in plasma exposure for either 5-CL, 5,7-DCL, or any metabolites. Female rats typically had higher concentrations of 5-CLG after administration of the two lower doses, but not at the 450 mg/kg/day dose.

Given the small sample size and moderate pharmacokinetic variance, no conclusion can be drawn regarding the linearity of halquinol pharmacokinetics in rats in the 50 – 450 mg/kg/day dose range. For 5,7-DCL, dose-normalized drug exposure (AUC) and C_{max} were proportional within the dose range administered (Figure 1). However, for dose-normalized AUC there was deviation from linearity for specific metabolites at the 150 mg/kg/day dose. A trend towards decreasing dose-normalized C_{max} with increasing dose was also noted for some metabolites.

Figure 1. Dose proportionality of halquinol in rats. (Bentz, 2015)

Dogs

As part of a GLP-compliant 13-week toxicity study (El Amrani-Callens, 2016), four groups of 8 Beagle dogs (4 male and 4 female)/group each received daily administration of the test item, Halquinol BP 80. The halquinol was administered as oral capsules at doses of 3, 10, 50, and 150 mg/kg bw/day. Dogs were fasted prior to dosing, with feed offered no earlier than one hour post-dose.

Blood samples were collected at 0, 1, 2, 4, 6 and 24 h following the first dose, and at the same time points following the last dose at the end of the 13-week treatment period. The concentrations of parent halquinol (5-CL and 5,7-DCL) and glucuronide and sulfate metabolites were determined in plasma using a validated LC-MS/MS method (Decorde, 2017). Noncompartmental kinetic analysis was performed to derive the PK parameters.

5-CL and its metabolites were only intermittently detected in canine plasma samples. 5-CL was not detected in any plasma samples in the 3, 10, and 60 mg/kg/day groups, and in only one plasma sample from one animal in the 150 mg/kg/day dose group. For the 5-CL metabolites, plasma concentrations were only detectable in the 1h sample at 3 mg/kg/day, but were detectable for increasing durations as the dose increased (detected up to 24 h post-dose for the 150 mg/kg/day group). Median time to maximum plasma concentrations (t_{\max}) was achieved 1–2 h post-administration for both 5-CLG and 5-CLS at all dose levels (Table 3). Combining both the Day 0 and Week 13 PK results, the mean peak concentrations (C_{\max}) of 5-CLG were approximately 500, 2700, and 6000 ng/mL (10, 60, and 150 mg/kg/day, respectively). Unlike the rat toxicokinetic results, peak 5-CLS sulfate concentrations were higher than the corresponding glucuronide metabolite by a factor of 1–2x. Combined (D1-Wk13) peak concentrations of 5-CLS were approximately 350, 950, 4340, and 5800 ng/mL for the 3, 10, 60, and 150 mg/kg/day dose groups, respectively.

Table 3. Summary of mean* pharmacokinetic parameters after halquinol oral administration in dogs (El Amrani-Callens, 2016)

Dose (mg/kg/ d)	Period	Parameter	Unit	5-CL	5-CLG	5-CLS	5,7-DCL	5,7-DCLG	5,7-DCLS
3	Day 1	t _{max}	h	NA	NA	1	1	NA	2
		C _{max}	ng/mL	NA	NA	337 (7)	196 (8)	NA	863 (7)
		AUC _{0-t}	ng*h/mL	NA	NA	NA	541 (2)	NA	2892 (1)
	Wk 13	t _{max}	h	NA	NA	1 (4)	2	NA	2
		C _{max}	ng/mL	NA	NA	363 (4)	134 (8)	NA	862 (8)
		AUC _{0-t}	ng*h/mL	NA	NA	NA	NA	NA	2052 (2)
	Day 1	t _{max}	h	NA	1	1	2	1.5	2
		C _{max}	ng/mL	NA	447 (7)	887 (8)	454 (8)	350 (2)	1566 (8)
		AUC _{0-t}	ng*h/mL	NA	NA	2430 (3)	1159 (7)	NA	3926 (6)
	Wk 13	t _{max}	h	NA	1	1	2	2	2
C _{max}		ng/mL	NA	565 (8)	1003 (8)	363 (8)	302 (5)	2168 (8)	
AUC _{0-t}		ng*h/mL	NA	1188 (1)	2344 (5)	1081 (7)	NA	6820 (7)	
10	Day 1	t _{max}	h	NA	2	1	2	2	2
		C _{max}	ng/mL	NA	2457 (8)	4352 (8)	1886 (8)	769 (8)	4604 (8)
		AUC _{0-t}	ng*h/mL	NA	5383 (7)	13316 (8)	5385 (8)	2162 (4)	16024 (8)
	Wk 13	t _{max}	h	NA	1.5	1	2	3	3
		C _{max}	ng/mL	NA	3024 (8)	4324 (8)	1815 (8)	1296 (8)	7413 (8)
		AUC _{0-t}	ng*h/mL	NA	6147 (8)	10752 (8)	5438 (8)	4077 (5)	23845 (8)
	Day 1	t _{max}	h	2	2	2	3	4	2
		C _{max}	ng/mL	36 (1)	4255 (8)	5733 (8)	1959 (8)	2682 (7)	6055 (8)
		AUC _{0-t}	ng*h/mL	NA	10410 (8)	13858 (8)	6428 (8)	10337 (4)	31061 (8)
	Wk 13	t _{max}	h	1	2	2	2	4	2
C _{max}		ng/mL	27.3 (1)	7744 (7)	5874 (7)	1511 (7)	2573 (7)	6674 (7)	
AUC _{0-t}		ng*h/mL	NA	23314 (7)	22753 (7)	5230 (7)	14282 (5)	46318 (7)	

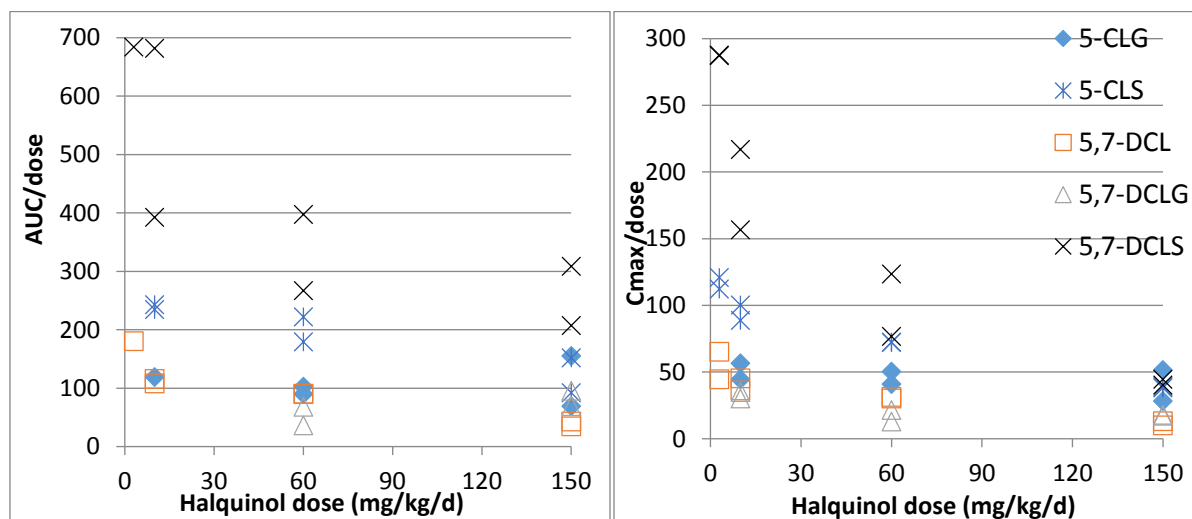
5,7-DCL was intermittently detected in canine plasma samples, but was quantified in at least one sample from all animals (including the lowest 3 mg/kg/day dose group). The glucuronide metabolite was not detected in any samples from the lowest dose group, but was quantifiable in at least some plasma samples from doses 10 mg/kg/day and greater. As with the 5-CL metabolites, plasma concentrations for 5,7-DCL metabolites were detectable for longer durations with increasing dose. Median time to maximum plasma concentrations (t_{\max}) was achieved 2–4 h post-administration for both 5,7-DCLG and 5,7-DCLS at all dose levels (Table 3). Combining both the Day 0 and Week 13 results, the mean peak concentrations (C_{\max}) of 5,7-DCL were approximately 165, 400, 1850, and 1735 ng/mL for the 3, 10, 60, and 150 mg/kg/day groups, respectively. Unlike the rat toxicokinetic study results, peak concentrations of 5,7-DCLG were typically lower than parent 5,7-DCL, except for the highest dose group. But similar to the rat study, concentrations of 5,7-DCLS predominated and were higher than 5,7-DCL and 5,7-DCLG at all time points.

Insufficient data were available to statistically evaluate the potential effect of gender on halquinol pharmacokinetics in dogs. No gender effect was consistently apparent when comparing concentrations of 5-CL, 5,7-DCL, or their metabolites in dogs.

No significant pharmacokinetic pattern was noted after multiple dosing (daily for 13 weeks) compared to the initial PK parameters derived after the first dose. Accumulation of halquinol parent compounds or metabolites in plasma was not readily apparent after repeated dosing.

The pharmacokinetics of halquinol in dogs were generally linear at the lower 3–10 mg/kg/day dose range (Figure 2). However, evidence of nonlinear kinetics appeared at higher doses (60 and 150 mg/kg/day). At these doses, the exposure (AUC) and peak amounts (C_{\max}) of 5,7-DCL, 5,7-DCLS, and 5-CLS decreased relative to the dose administered. Conversely, the limited data displayed a trend of increased 5,7-DCLG exposure relative to the dose in the highest dose group. This potentially indicates saturation of the 5,7-DCLS metabolic pathway in dogs after large dose administration, with subsequent shunting to the glucuronide pathway. However, no definite conclusions can be drawn given the limited sample size and moderate inter-animal PK variance.

Figure 2. Dose proportionality of halquinol in dogs (El-Amrani-Collins, 2016)



Mini-pigs

In a GLP-compliant study (Chevalier, 2014), three groups of Göttingen minipigs (n=3 male and 3 female per group) received Halquinol BP 80 by oral gavage at 25, 75 or 225 mg/kg bw/day for 4 weeks. Plasma was drawn at 2 h post-treatment on day 26; levels of 5-CL, 5,7-DCL, and their sulphate and glucuronide metabolites were determined using a non-validated LC-MS/MS method.

PK parameters were not determined due to the limited plasma sampling. 5-CL was not quantifiable in plasma samples from any dose group. 5-CLG was the primary metabolite produced from 5-CL, with 5-CLS only detectable in the 225 mg/kg dose group. 5,7-DCL was detected in plasma of all animals. The 5,7-DCLG concentration was typically 2 – 5x higher than parent 5,7-DCL, with 5,7-DCLS concentrations generally lower (25-125 %) than parent 5,7-DCL. Due to the small number of minipigs in the study and plasma samples collected, no conclusions regarding gender effect or dose linearity could be made.

Pharmacokinetics in Food-producing Animals

Pigs

In a non-GLP-compliant study (Swan 2016), four male (castrated) and four female (gilt) pigs received oral halquinol by one of two dose regimens: a single oral dose of 12 mg/kg administered as a 100 mg/mL halquinol suspension in 0.5 % methylcellulose, or a 10-day dosage regimen comprised of 6 mg/kg bid (12 mg/kg/day) using the same halquinol suspension. This dose regimen is midway between the approved lower and upper dose regimens for Quixalud® in pigs (2.4 – 24 mg/kg/day). The same commercial-bred pigs were used in both studies, with a washout period of 14 days between them. The total volume of all oral doses was < 5 mL, and was orally gavaged at the back of the throat followed by gavage of 5-10 mL of water. Pigs were fed a non-medicated diet *ad libitum*. All pigs weighed approximately 8 kg upon arrival.

Blood was collected at the following time points during the study:

- Single-dose portion: 0, 0.5, 1, 2, 4, 6, 8, 12, 16, 24, 36, 48, 60, and 72 hr post-dosing;
- Multiple dose portion (blood collected from only 3 male and 3 female pigs):
 - 0, 0.5, 1, 2, 4, 6, 8, 12, 16, and 24 hr post-dosing on Day 0;
 - A single blood sample prior to the first dose on days 2, 3, 4, 5, 7 and 10;
 - 0, 0.5, 1, 2, 4, 6, 8, 12, 16, 24, 36, 48, 60, and 72 h post final dose on day 10.

The concentrations of parent halquinol components (5-CL and 5,7-DCL) and their glucuronide and sulfate metabolites were to be determined in plasma using a validated LC-MS/MS method (Scheele, 2015). However, the transferred method was not validated; and the revised method with a lower LLOQ was not fully developed. The sponsor indicated that this deviation had a minor impact on the study.

Noncompartmental kinetic analysis was performed to derive the PK parameters. In the single 12 mg/kg oral dose study, no plasma samples had 5-CL concentrations above the LLOQ of 9.0

ng/mL. The other parent component, 5,7-DCLG, was quantifiable in plasma (LLOQ 45.0 ng/mL).

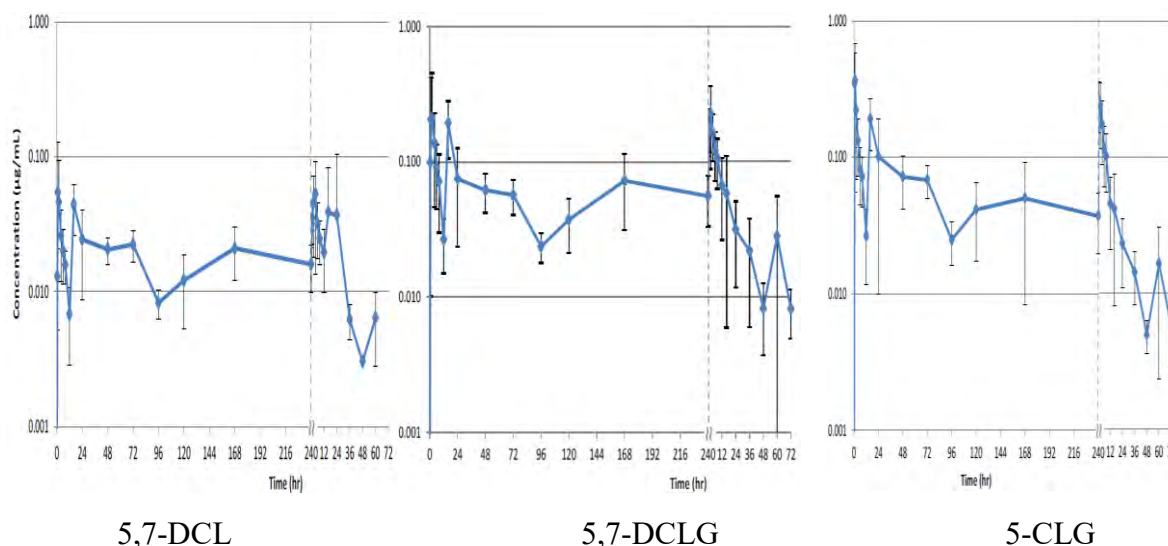
The glucuronide forms (5-CLG and 5,7-DCLG) were the primary halquinol metabolites after single-dose administration with quantifiable results for most pigs. Although the 5-CL isomer could not be detected in systemic circulation, the 5-CLG metabolite (as well as corresponding 5,7-DCLG) were rapidly produced (t_{\max} 0.5–6 h). Detection of the glucuronide form of both compounds indicates that absorption of both the 5-CL and the 5,7-DCL components occurred, with rapid metabolism to the glucuronide form.

Limited quantifiable concentrations of the sulfate metabolites (5-CLS and 5,7-DCLS) were observed. For 5-CLS, only 3 pigs had plasma concentrations above the LLOQ of 225 ng/mL (at a single time point, 0.5 h post-dose). For 5,7-DCLS, only 1 pig (at 2 time points) had plasma concentrations above the LLOQ of 225 ng/mL.

Limited pharmacokinetic analysis could be performed for 5-CL, 5-CLS, and 5,7-DCLS due to lack of quantifiable plasma results. For the 5-CLG metabolite, t_{\max} occurred between 0.5–1 h with a C_{\max} range of 552 – 2960 ng/mL. The t_{\max} range of 5,7-DCL was 1–6 h post-dose, with a C_{\max} range of 67–494 ng/mL. For 5,7-DCLG, the t_{\max} occurred between 0.5–6 h post dose, with plasma concentrations comparable to 5-CLG. The C_{\max} for 5,7-DCLG was approximately 4x higher than parent 5,7-DCL (range 242–1770 ng/mL).

In the multiple dose portion of the study, again only the 5,7-DCL component and both glucuronide metabolites (5-CLG and 5,7-DCLG) were quantifiable in any plasma samples. The LLOQs for the non-quantifiable analytes were 9.00 ng/mL (5-CL) and 225 ng/mL (both sulfate metabolites). Because only a small number of 5,7-DCL, 5-CLG, and 5,7-DCLG samples were quantifiable using the initial method (LLOQ 45 ng/mL), a revised analytical method (LLOQ 3.0 ng/mL) was developed and the samples re-analysed. However, the QC samples that were part of the reanalysis showed considerable variability from nominal. Therefore, the sample results were not considered quantitatively reliable and thus were only considered to be qualitative. No pharmacokinetic analyses were conducted on the Cycle 2 data, but plasma concentrations over time are shown in Figure 3.

Figure 3. Plasma concentrations of 5,7-DCL, 5,7-DCLG, and 5-CLG in pigs receiving halquinol at 12 mg/kg/day. (Swan, 2016)



Summary of PK data – all species

Direct comparisons of halquinol pharmacokinetic parameters (C_{max} and AUC) cannot be made between species based on the data provided. This is due to significant differences in halquinol dose ranges used, methods of oral administration, plasma sampling schedules, and assay characteristics (LLOQ) for each study. However, despite these challenges, a number of observations were common to all studies:

- 5-CL concentrations were non-detectable in all but one plasma sample from all studies combined, even after repeated and/or supra-therapeutic (> 25 mg/kg/day) doses of halquinol. As the glucuronide and/or sulfate metabolites were rapidly detected in plasma after halquinol administration in all species studied, it is presumed that 5-CL is absorbed from the GI tract and undergoes extensive first-pass metabolism.
- 5,7-DCL was detectable after oral halquinol administration in all species tested, even at < 25 mg/kg/day (therapeutic dose range in pigs). Peak plasma concentrations were typically reached a few hours after halquinol administration, and then declined rapidly (typically quantifiable for 4–12 h post-dose).
- Significant bioaccumulation after repeated dosing was not observed in any species. Plasma concentrations of parent halquinol or the glucuronide/sulfate metabolites were not increased after prolonged daily dosing regimens.
- There appeared to be no significant gender effects on halquinol pharmacokinetics in any species, though the sample sizes for all studies were too small to draw definite conclusions.

- The linearity of halquinol pharmacokinetics cannot be conclusively determined based on dose ranges used in the dog and rat studies. A trend towards decreasing halquinol exposure with increasing dose was observed in both rats and dogs, but only at supra-therapeutic doses (beyond 50 mg/kg/d). At the therapeutic dose range in pigs (2.4 – 24 mg/kg/d), halquinol pharmacokinetics were linear in dogs.
- Note that the methods of oral administration in all studies differ from the approved halquinol formulation (mixed in feed). The PK studies administered halquinol via oral gavage, either as a methylcellulose gel or in capsule form. It is not known if concurrent feed intake alters halquinol kinetics in the target species.
- Oral bioavailability of halquinol was not evaluated in any species.

Differences in halquinol metabolism between species are apparent. Overall metabolite exposure (AUC) data is not available for all metabolites in all species, due to differences in dose levels, plasma sampling times, and assay sensitivity between studies. However, using maximum plasma concentrations (C_{\max}) of the various halquinol metabolites as a rough estimate of metabolite production, and normalizing the results based on dose, certain trends are observed (Table 4).

- For 5-CL, the glucuronide metabolism pathway predominates in the rat and pig, whereas the sulfate pathway predominates in the dog (at least until very high doses of halquinol are administered, at which point increasing ratios of glucuronide: sulfate metabolites were observed).
- For 5,7-DCL, only the glucuronide pathway produced quantifiable metabolites in the pig. However, the sulfate pathway predominates for 5,7-DCL in the dog, and both pathways produce roughly comparable metabolite concentrations in the rat.

Table 4. Comparison of mean halquinol C_{\max} and dose-adjusted C_{\max} by species and dose.

Species	Dose (mg/kg/d)	Period	5-CL		5-CLG		5-CLS		5,7-DCL		5,7-DCLG		5,7-DCLS	
			C_{\max}	Dose-adj.	C_{\max}	Dose-adj.	C_{\max}	Dose-adj.	C_{\max}	Dose-adj.	C_{\max}	Dose-adj.	C_{\max}	Dose-adj.
Dog	3	Day 0	ND	NA	NA	NA	337	112.3	196	65.3	NA	NA	863	287.7
		Wk 13	ND	NA	NA	NA	363	121.0	134	44.7	NA	NA	862	287.3
	10	Day 0	ND	NA	447	44.7	887	88.7	454	45.4	350	35.0	1566	156.6
		Wk 13	ND	NA	565	56.5	1003	100.3	363	36.3	302	30.2	2168	216.8
	60	Day 0	ND	NA	2457	41.0	4352	72.5	1886	31.4	769	12.8	4604	76.7
		Wk 13	ND	NA	3024	50.4	4324	72.1	1815	30.3	1296	21.6	7413	123.6
	150	Day 0	36	0.2	4255	28.4	5733	38.2	1959	13.1	2682	17.9	6055	40.4
		Wk 13	27.3	0.2	7744	51.6	5874	39.2	1511	10.1	2573	17.2	6674	44.5
Rat	50	Day 0	ND	NA	2913	58.3	818	16.4	245	4.9	2607	52.1	3009	60.2
		Wk13	ND	NA	2794	55.9	2521	50.4	96	1.9	2878	57.6	6502	130.0
	150	Day 0	ND	NA	4304	28.7	1616	10.8	800	5.3	4187	27.9	4849	32.3
		Wk 13	ND	NA	4614	30.8	5055	33.7	518	3.5	3933	26.2	7889	52.6

Minipig	25	Day 26	ND	NA	2711	108.4	ND	NA	1308	52.3	4498	179.9	867	34.7
	75	Day 26	ND	NA	6588	87.8	ND	NA	2534	33.8	9467	126.2	1232	16.4
	225	Day 26	ND	NA	2963 3	131.7	321	1.4	8086	35.9	2876 9	127.9	3276	14.6
Pig*	12	Single	ND	NA	963	80.2	ND	NA	177	14.8	648	54.0	ND	NA
		Day 0	ND	NA	705	58.8	ND	NA	132	11.0	428	35.7	ND	NA
		Day 10	ND	NA	326	27.2	ND	NA	63	5.3	345	28.8	ND	NA

*Note: For pig data, original PK results from the less sensitive (LOQ 225 ng/mL) but validated assay were used, as the revised sample results were considered unreliable.

Metabolism in laboratory animals

While no traditional *in vivo* metabolism studies have been conducted in laboratory animals by the Sponsor, there are examples of halquinol and same class molecule metabolism in laboratory animals referenced in the literature.

In a balance-excretion study in male Wistar rats (Bories & Tulliez, 1972), a mixture of 5,7-DCL and 5-CL, labeled with [³⁶Cl] (dose = 3.75 mg, ~15 mg/kg bw) was dissolved in peanut oil and administered orally by stomach intubation. In a second experiment, rats were administered labeled drug orally in the feed at the same dose (equivalent to 250 ppm in the feed). Approximately 90 % of the radioactivity in rats had been excreted in the urine or feces within 48 h, feces being the predominant route of elimination. The authors reported elimination as the unmetabolized parent, conjugate forms (undistinguished between sulfate and glucuronide), and limited dechlorinated form.

In Hayashi, *et.al.*, (1976), urinary and biliary excretion was examined in rats, guinea-pigs and rabbits. Male albino rats were administered iodochlorohydroxyquin by oral gavage, as a 1.5 mL 0.32 % w/v sodium carboxymethyl cellulose (CMC-Na) suspension (dose = 15 mg, ~ 50 mg/kg bw). In rats, iodochlorohydroxyquin sulfate was the primary metabolite excreted in the urine with smaller amounts of glucuronide and trace amounts of unmetabolized parent detected in the urine. The same suspension of iodochlorohydroxyquin was administered orally in the rat with bile fistula; glucuronide conjugate was almost exclusively present in the bile, with little sulfate conjugate or unmetabolized parent detected. In plasma, the sulfate conjugate was detected at 1.5–2 times higher concentrations than the glucuronide metabolite; only trace amounts of the unmetabolized parent were present. In male guinea-pigs, iodochlorohydroxyquin was administered by oral gavage as the same CMC-Na suspension as per the rat experiment (dose = 24 mg/animal, ~ 60 mg/kg bw). The glucuronide metabolite was the primary metabolite detected in urine. Small amounts of the sulfate conjugate were detected in urine, and only trace amounts of the unmetabolized parent were detected. In plasma, the glucuronide conjugate was detected at higher concentrations than the sulfate metabolite; and only trace amounts of the unmetabolized parent were present.

The more recent toxicokinetic and pharmacokinetic data in laboratory animals and pigs demonstrates rapid metabolism of parent halquinol components to their glucuronide and sulfate conjugate forms. The relative proportions of glucuronide versus sulfate metabolites are not uniform, but vary depending on species, dose, and parent halquinol component. A simplistic

summary of metabolite formation (based on maximum plasma concentrations) is shown in Table 5.

Table 5. Relative prevalence of halquinol conjugates in plasma by species.

Species	5-CL	5,7-DCL
Rat	G > S	S > G
Dog	S > G	S > G
Minipig	G > S	G > S
Pig	G > S	G > S

Metabolism in Food-producing Animals

Pigs

In a GLP compliant study (McLean, 2016), four groups of 2 male and 2 female 8-week old swine, weighing 17.2 to 23.2 kg received 12 mg [¹⁴C]-halquinol/kg bw/d for 7 consecutive days. The dose was administered in gelatin capsules twice daily, approximately every 12 h, such that half of the daily dose (6 mg [¹⁴C]-Halquinol/kg body weight/dose) was administered in each capsule. The dose was estimated to be approximately one-half of the maximum label dose, assuming a feed consumption rate for pigs of this age at 4 % of body weight. The benzene ring was uniformly labelled for both halquinol components. The radiochemical purity of the test article was 99.3 % with a specific activity of 2.7 µCi/mg. Animals in group 4 were individually housed in metabolism crates and urine and feces were collected every 24 h until slaughter. A final cage wash also was collected following removal of animals from the metabolism crates. Animals in treatment groups 1-4 were slaughtered at 3, 6, 12 and 48 h, respectively, post-final dose. Liver, kidneys, loin muscle, skin with fat, bile, heart and small intestine were collected. All samples were analysed by combustion, LSC and HPLC. Table 6 summarizes the total radioactive recovery (as % total dose administered) in the urine, feces, and cage wash from each animal.

Table 6. Total Radioactive Recovery of [¹⁴C]-halquinol (as % total dose administered) in the urine, feces and cage wash of Group 4 Animals.

Sample	Total Radioactive Recovery (as % total dose)			
	13M	14M	15F	16F
Feces	34.6	35.6	36.2	37.8
Urine	53.5	56.0	31.2	25.1
Cage Wash	0.9	2.2	3.1	2.9
Total	89.1	93.85	70.4	65.8

The highest radioactive recovery was found in males, compared to females. The dose was rapidly excreted with a mean 80 % of the dose recovered within 48 h of the last dose. The cumulative excretion of radioactivity in feces was similar in all animals, with a mean excretion of 36 % of the total dose administered. The cumulative excretion of radioactivity in urine was higher in males than in females, with a mean recovery of 55 % in males compared to a mean

recovery of 28 % in females. The total radioactive recovery of [^{14}C]-Halquinol in the cage wash was low, with a mean recovery of 2.3 %.

The recovery of radioactivity in tissues was very low. Liver contained the highest recovery, ranging between 0.008 to 0.107 %. Kidneys ranged between 0.002 to 0.044 %. The lowest radioactive recovery was found in the loin muscle and skin with fat samples which both had 0 % recovery of the total dose administered across all animals. Selected edible tissues (liver, kidney, muscle and skin with fat) were subject to serial solvent extraction and subsequent quantification of the extractable residues. Table 7 provides a summary of the distribution of extractable and non-extractable radioactivity in tissues, expressed as a percent of the TRR.

Table 7. Distribution of extractable and non-extractable radioactivity in tissues, expressed as % of the total radioactive residues (TRR).

Tissue	Animal ID	Withdrawal Period (h)	Extractable Radioactivity (% TRR)	Non-extractable Radioactivity (% TRR)
Liver	1M	3	50.7	49.3
	4F	3	26.8	73.2
	6M	6	37.4	62.6
	8F	6	37.0	63.0
	10M	12	24.0	76.0
	11F	12	18.3	81.7
Kidney	1M	3	87.6	12.4
	4F	3	62.2	37.8
	6M	6	81.0	19.1
	8F	6	73.4	26.6
	10M	12	43.2	56.8
	11F	12	32.5	67.5
	13M	48	15.9	84.1
	16F	48	25.2	74.8
Muscle	1M	3	81.6	18.4
Skin with Fat	1M	3	85.6	14.5

Extractability was higher in muscle and skin with fat (81.6 % and 85.6 % TRR, respectively) at the 3-hour time point. Extractability was lower in the liver and kidney (18.3 % to 50.7 % and 15.9 % to 87.6 %, respectively). By 12 h, extractability in the kidney decreased. Although the extractability in kidney and liver decreased at later times, the concentration of TRR continued to decline over time, indicating that the product was continuing to be eliminated.

The quantitative distribution of radioactive residues was assessed by HPLC in urine, feces, 6 liver, 4 kidney, one muscle, and one skin with fat samples. Glucuronide metabolites accounted for 83 % of the urine TRR and unchanged parent accounted for 13 %. Unchanged parent accounted for 12 % of the TRR and 100 % of the extractable residues in feces. 5-CL was not detected in liver, kidney, muscle or skin with fat. 5,7-DCL was detected and quantified in liver, kidney, muscle and skin with fat. The metabolite 5-CLG was confirmed in liver, kidney, muscle

and skin with fat. 5,7-DCLG was confirmed in kidney, muscle and skin with fat. An additional component was identified in liver; however, it could not be identified and was a suspected source fragment from the mass spectrometer.

Table 8 lists the quantitative distribution of radiolabelled residues present in the tissues analysed. In liver, the radiolabeled halquinol and its glucuronide metabolites accounted for only 1 – 5 % of the total radioactivity present. Other uncharacterized metabolites were detected, but the sum of all extracted radiolabeled components in liver accounted for only 18 – 42 % of the total tissue radioactivity. In kidney, halquinol and its glucuronide metabolites accounted for 25 – 52 % of the total tissue radioactivity. Including other uncharacterized metabolites, the sum of all extracted radiolabeled components in kidney accounted for ≤ 70 % of the total radioactive residues. The identity of the uncharacterized residues in liver and kidney could not be ascertained based solely on the chromatograms. In muscle and skin with fat, halquinol and its glucuronide metabolites were the only radiolabelled components detected, and accounted for 60 and 73 % of total tissue radioactivity, respectively. However, only one sample was analysed for each of muscle and skin with fat, as the total tissue radioactivity was extremely low in all other muscle and skin with fat samples.

Table 8. Quantitative distribution of radiolabelled halquinol components in various swine tissue expressed as percentage of total and extractable radioactive residues (TRR/ERR).

Tissue	Animal	WP	5-CLG % TRR (% ERR ¹)	5,7-DCLG % TRR (% ERR)	5-CL % TRR (% ERR)	5,7-DCL % TRR (% ERR)	Total MR as % TRR ²	Total individual components as % TRR ³
Liver	1M	3	3.6 (8.7)	ND	ND	1.4 (3.5)	5.0	41.7
	4M	3	2.3 (8.8)	ND	ND	2.5 (9.6)	4.8	25.8
	6M	6	3.3 (10.1)	ND	ND	0.8 (2.6)	3.9	33.1
	8F	6	2.1 (7.7)	ND	ND	2.2 (8.1)	4.3	27.2
	10M	12	1.3 (5.4)	ND	ND	0.7 (2.9)	2.0	24.0
	11F	12	ND	ND	ND	1.4 (7.4)	1.4	18.3
Kidney	1M	3	20.8 (29.7)	ND	ND	6 (8.5)	26.8	70.4
	4F	3	15.3 (28.7)	ND	ND	9.9 (18.6)	25.2	53.1
	6M	6	25 (35.4)	22.6 (31.9)	0.6 (0.8)	5 (7.1)	53.2	70.7
	8F	6	22.5	ND	ND	5.7	28.2	58.5
Muscle	1M	3	18.8 (31.1)	37.2 (61.4)	ND	4.5 (7.5)	60.5	60.5
Skin/fat	1M	3	17.3 (23.4)	23.8 (32.2)	ND	32.7 (44.4)	73.8	73.8

¹% ERR based on extractability of the final extractions used for the HPLC analysis, and differs slightly from the extractability listed in Table 7.

²Proposed marker residue = sum of 5-CL, 5,7-DCL, 5-CLG, and 5,7-DCLG

³Sum of all individual components (marker residue and other uncharacterized metabolites on chromatogram) as a percentage of TRR

ND = not detected

Because of the quick rate of metabolism and limited presence of parent halquinol, the marker residue is proposed by the sponsor to be the sum of 5-CL, 5,7-DCL, 5-CLG (expressed as 5-CL-equivalents), and 5,7-DCLG (expressed as 5,7-DCL-equivalents).

Comparative metabolism

In a GLP compliant study (Novo, 2015), cryopreserved hepatocytes and hepatic microsomes prepared from male and female Sprague Dawley rats, beagle dogs, Goettingen minipigs, Landrace pigs and humans were incubated with 5 and 20 μM [^{14}C]-5,7-DCL. The rate of metabolism was determined by subjecting samples to LC-MS analysis with online radiodetection. The viability of each hepatocyte preparation was assessed using a Trypan blue exclusion assay and determined to be between 67 and 97 %.

[^{14}C]-5,7-DCL was extensively metabolized in all species and genders. Complete metabolism was observed in hepatocytes from male and female minipigs and pigs. Complete metabolism also was observed in microsomes from male and female dogs and pigs and female minipigs where no [^{14}C]-5,7-DCL was detected at the end of the incubation period. The lowest rates of metabolism were observed in rat male and female microsomes, which showed 19.6 and 26.8 % total metabolism, respectively. However, the rates of metabolism in rat hepatocytes was high (92.6 and 86.7 % in male and female, respectively), suggesting a preference for Phase II metabolism of [^{14}C]-5,7-DCL in this species.

LC-MS analysis was used to determine the structural identity of the metabolites formed in the high dose (20 μM) hepatocyte and microsome samples (see Table 9). In hepatocytes of all species, one metabolite of [^{14}C]-5,7-DCL was detected: a glucuronide conjugate (M2). A glucose conjugate (M1) was detected in hepatocytes of all species, except humans. Dechloro hydroxyl conjugates of [^{14}C]-5,7-DCL (2 isomers; M4) also were detected in hepatocytes of minipigs and pigs. In hepatic microsomes, one metabolite, oxidized parent compound (M3) was identified in all species, except rats. One additional peak at approximately 14 minutes was present in some hepatic microsome samples (dogs and minipigs), but could not be identified. The results of the study demonstrate that the metabolic profiles of 5,7-DCL in Sprague Dawley rats, dogs, minipigs, pigs, and humans are qualitatively similar.

Table 9. Metabolites identified by LC-MS from incubation of hepatocytes and microsomes from rats, dogs, minipigs, pigs and humans with 20 μM [^{14}C]-5,7-dichloroquinolin-8-ol.

	Glucose Conugate (M1)	Glucuronide Conjugate (M2)	Oxidized Parent (M3)	Dechloro hydroxyl conjugates (2 isomers; M4)	Unidentified Peak at ~ 14 min
Hepatocytes					
Sprague- Dawley Rat	+	+	-	-	-
Beagle Dog	+	+	-	-	-
Goettingen Minipig	+	+	-	+	-
Landrace Pig	+	+	+	+	-
Human	-	+	-	-	-
Microsomes					
SD Rat	-	-	-	-	-
Beagle Dog	-	-	+	-	+

Goettingen Minipig	-	-	+ (male)	-	+
Landrace Pig	-	-	+	-	-
Human	-	-	+	-	-

Of note, no sulfate conjugates of 5,7-DCL were observed in this *in vitro* study. 5,7-DCLS was not observed in pharmacokinetic studies in swine, though it was the predominant 5,7-DCL metabolite identified in dog and rat pharmacokinetic studies. The authors hypothesized that lack of observed sulfate conjugates may be the results of 5,7-DCL not being a substrate of sulfoltransferase enzymes *in vitro*, or the incubation conditions strongly favoring glucuronidation as a conjugation pathway.

On the basis of the experimental observations, the following biotransformation pathways are proposed for 5,7-DCL and 5-CL (Figures 4 and 5, respectively). The biotransformation pathway for 5,7-DCL represents any metabolite that was detected *in vitro*, as well as any known metabolites (e.g. sulfate) detected in the toxicology and pharmacokinetic studies performed in pigs, rats, dogs and minipigs. The biotransformation pathway for 5-CL only represents the known metabolites detected in the toxicology and pharmacokinetic studies conducted in pigs, rats, dogs and minipigs (5-CL was not included in the *in vitro* testing).

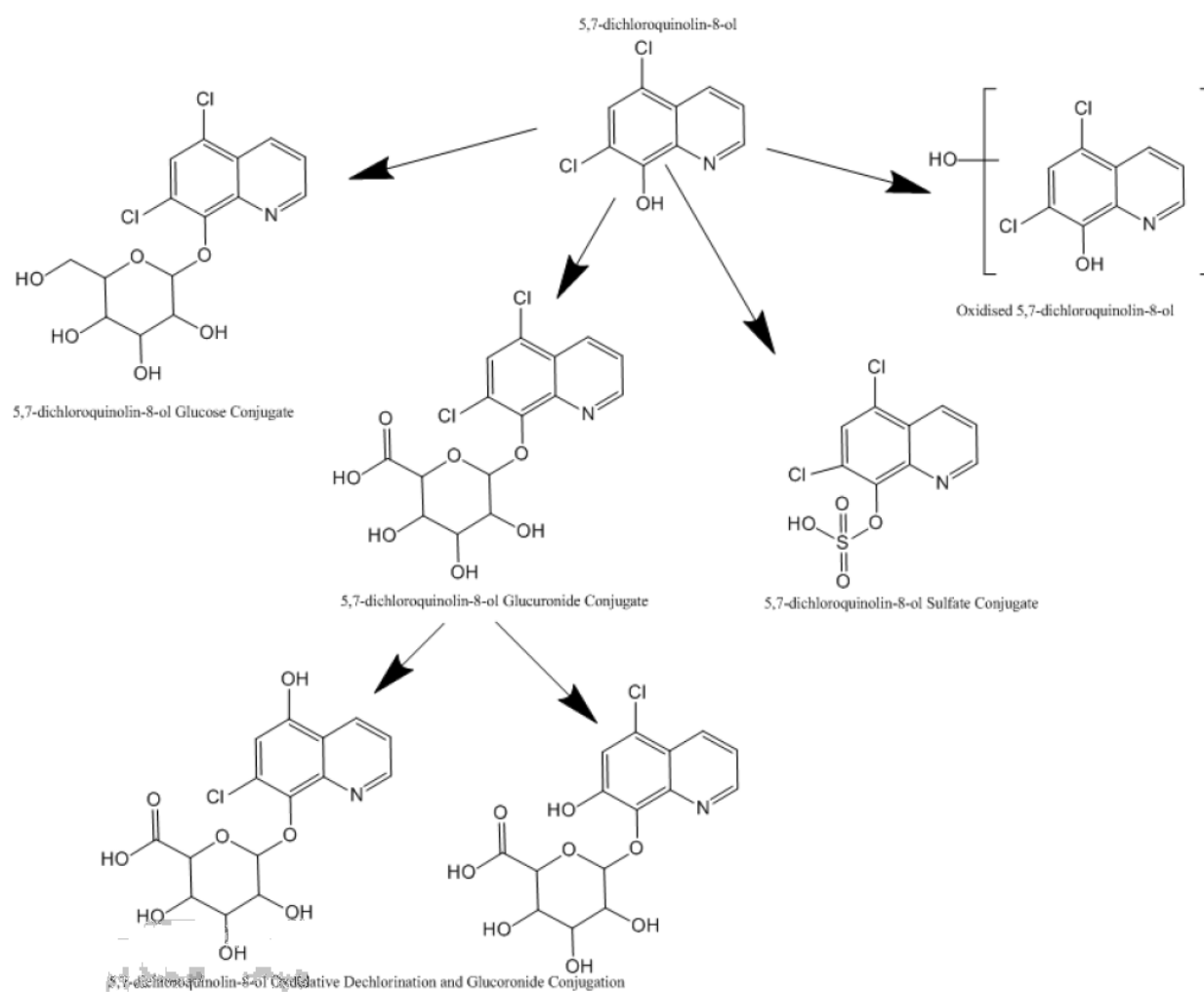
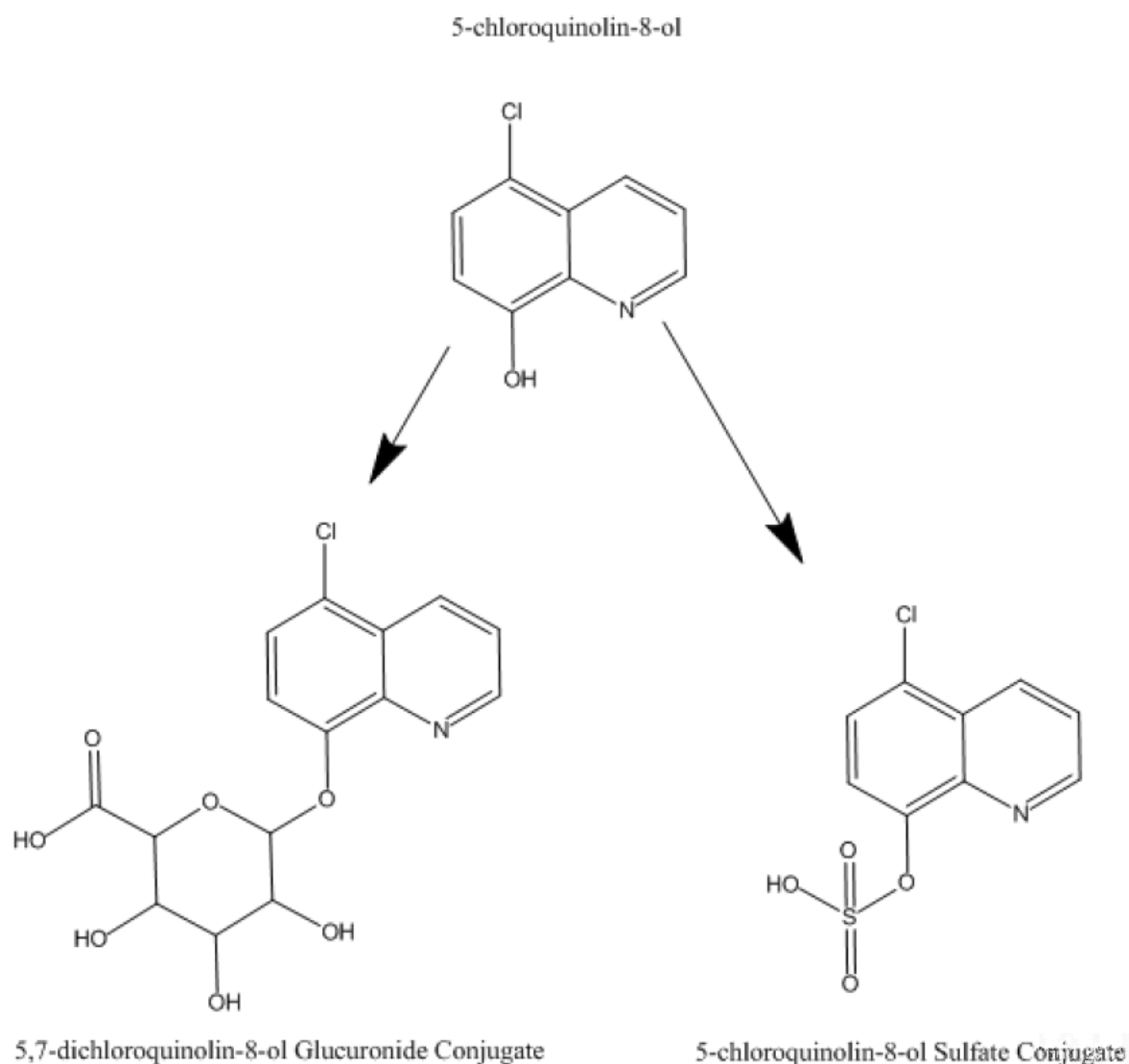
Figure 4. Biotransformation pathway for 5,7-DCL.

Figure 5. Biotransformation pathway for 5-chloroquinolin-8-ol.

Tissue residue depletion studies

Radiolabelled residue depletion studies

Pigs

In a GLP-compliant study (McLean, 2016), four groups of 2 male and 2 female 8-week old swine, weighing 14.3 to 19.4 kg received 12 mg [^{14}C]-Halquinol/kg body weight/day for 7 consecutive days. The dose was administered in gelatin capsules twice daily, approximately every 12 h, such that half of the daily dose (6 mg [^{14}C]-Halquinol/kg body weight/dose) was administered in each capsule. Capsules were administered along with bread, and animals had access to food at the time of capsule administration in order to mimic the fed state (as per label dosing instructions in feed). The dose was estimated to be approximately one-half of the maximum label dose, assuming a feed consumption rate for pigs of this age at 4 % of body weight. The benzene ring was uniformly radiolabelled for both components. Animals were slaughtered at 3, 6, 12 and 48 h, respectively, post-final dose. Liver, kidneys, loin muscle, skin

with fat, bile, heart and small intestine were collected and analysed for total radioactive residues using liquid scintillation and combustion. Mean TRR in edible tissues across the groups were highest in kidney, followed by liver, skin with fat and muscle (Table 10).

Table 10. Mean concentrations ($\mu\text{g/kg}$) of total radioactive residues (TRR) in tissues of swine dosed with [^{14}C]-halquinol at 12 mg/kg for 7 days.

Time Post-Dose (h)	Kidney TRR ($\mu\text{g/kg}$)	Liver TRR ($\mu\text{g/kg}$)	Muscle TRR ($\mu\text{g/kg}$)	Skin with Fat TRR ($\mu\text{g/kg}$)
3	3379	1605	81	286
6	3677	1063	53	155
12	1800	770	33	105
48	494	247	10	27

Because the proposed marker residue method was not fully developed or validated at the time of the radiolabelled study, the samples incurred from this 2016 study were transferred to GLP study NAH-016-077 and analysed using the validated LC-MS/MS method for the sum of halquinol residues (Ward, 2017a, 2017b). Two tissue samples (one male and one female) from each treatment group were analysed 440 days after collection for the components comprising the proposed halquinol marker residue (5-CL, 5,7-DCL, 5-CLG [expressed as 5-CL equivalents] and 5,7-DCLG [expressed as 5,7-DCL equivalents]). As these tissue samples had been frozen for 440 days between collection and analysis, the stability of halquinol-fortified frozen ($-80\text{ }^{\circ}\text{C}$) tissue samples was assessed in 2017 (unpublished data). After 191 – 239 days of storage ($-80\text{ }^{\circ}\text{C}$), the stability of halquinol in kidney was consistently below the acceptance range of 70 – 100 % of initial concentration. Stability of halquinol residues in frozen muscle samples was borderline ($\sim 70\text{ }\%$ of initial concentration). Note that the frozen stability of all incurred (as opposed to fortified) halquinol tissue residues from Hall 2017 did meet acceptance criteria. Due to the instability of frozen halquinol residues in kidney (and possibly muscle) tissues at 440 days, the halquinol concentrations in pig tissues from the 2016 McLean study, but derived using the 2017 Ward assay, are not considered valid for determining a marker residue: total residue. For comparison with the total radioactive residue concentrations determined in McLean 2016, the unvalidated halquinol marker residue concentrations of the same sample (analysed 440 days later using the Ward LC-MS/MS method) are listed in Table 11.

Table 11. Proposed halquinol marker residue concentrations (µg/kg) in select tissues of swine dosed with [¹⁴C]-halquinol at 12 mg/kg for 7 days.

Tissue	Animal	WP	TRR (McLean)	MR (McLean)	MR* (Ward)
Muscle	1M	3	163	99	89
Skin/fat	1M	3	552	406	393
Kidney	1M	3	5935	1592	3080
Kidney	4F	3	3657	922	1130
Kidney	6M	6	4022	2140	2130
Kidney	8F	6	4135	1165	1710
Liver	1M	3	2344	119	622
Liver	4F	3	2222	105	230
Liver	6M	6	671	28	146
Liver	8F	6	1575	68	299
Liver	10M	12	870	17	72
Liver	11F	12	985	13	28

*Concentrations not validated

The lack of frozen sample stability should have resulted in lower proposed marker residue concentrations as determined by the Ward LC-MS/MS assay. However, the liver (and some kidney) LC-MS/MS results were considerably higher. The sponsor proposed that halquinol residue is conjugated to macromolecules (potentially contributing to the low extractability of radioactive residues in liver and kidney), and that degradation of these conjugates upon long-term storage and/or sample processing for LC-MS/MS resulted in an apparent increase in marker residue concentrations.

Residue depletion studies with non-radiolabelled drug

Pigs

In a GLP compliant study, four groups of 2 male and 2 female 8-week old swine, weighing 19.8 to 28 kg were offered medicated feed containing 700 ppm halquinol *ad libitum* to achieve a targeted inclusion rate of 12 mg/kg for 10 consecutive days (Hall, 2017). Flavor enhancer and sweetener were added to the treated feed at 300 ppm. Two control animals (1 male and 1 female) were allowed *ad libitum* access to a non-medicated feed. Feed analysis results indicated the halquinol concentration ranged from 619 – 654 ppm in feed (nominal concentration 700 ppm) and was homogenous in all feed samples tested. The stability of the medicated feed was acceptable (> 80 % potency for the duration of the study) for 2/3 batches tested, and nearly acceptable (76 % potency) for the remaining batch.

Control animals were slaughtered at day -2. Treated animals were slaughtered at 8, 24, 48 and 120 h, respectively, post-final dose. Liver, kidney, loin muscle, skin with fat and small intestine were collected. All tissue samples except for small intestine were analysed for the proposed halquinol marker residue (sum of 5-CL, 5,7-DCL, and their glucuronide metabolites expressed as parent equivalents) using a validated LC-MS/MS method (Ward 2017a, 2017b).

The individual animal and mean concentrations of proposed halquinol marker residue are listed in Table 12. Halquinol marker residues depleted most slowly from the kidney. By 120 h withdrawal, residues only were detected in the kidney (4/4 samples) and skin with fat (1/4 samples).

Table 12. Individual animal and mean concentrations ($\mu\text{g/kg}$) of proposed halquinol marker residue (\pm s.d.) in tissues of swine dosed with 700 ppm halquinol for 10 days.

Withdrawal Period (h)	Muscle	Liver	Kidney	Skin with fat
8	29	210	1482	96
	33	299	918	118
	23	206	2569	96
	27	286	1780	90
Mean \pm s.d.	28 ± 4	250 ± 49	1687 ± 688	100 ± 12
24	<LOQ	66	230	48
	<LOQ	38	219	25
	<LOQ	45	1195	31
	<LOQ	26	192	21
Mean \pm s.d.		44 ± 17	459 ± 491	31 ± 12
48	<LOQ	<LOQ	65	<LOQ
	<LOQ	<LOQ	42	<LOQ
	<LOQ	<LOQ	20	<LOQ
	<LOQ	<LOQ	52	19
Mean \pm s.d.			45 ± 19	
120	<LOQ	<LOQ	21	<LOQ
	<LOQ	<LOQ	23	<LOQ
	<LOQ	<LOQ	108	16
	<LOQ	<LOQ	<LOQ	<LOQ
Mean \pm s.d.			51 ± 50	

An exponential regression on the log transformation of the tissue residues showed that the depletion half-lives for liver and kidney were approximately 9 and 21 h, respectively. The depletion half-life for skin with fat was approximately 11 h and the depletion half-life for muscle was approximately 7 h.

Determination of the Marker: Total (MR:TRR) Ratio

A dosing error was noted as being written into the protocol of the radiolabelled halquinol residue depletion study (McLean, 2016). In that study, animals should have received two daily doses of halquinol at 12 mg/kg, for a total of 24 mg/kg/day; rather than 6 mg/kg per dose, for a total of 12 mg/kg/day. The actual dose administered in the radiolabelled study was determined to be 12.4 mg/kg bw/piglet. In the non-radiolabelled residue depletion study (Hall, 2017), the total daily dose was 40.1 mg/kg due to higher than expected *ad libitum* medicated feed intake in study animals. To account for the discrepancy between the doses in the total residue and residue depletion studies, the sponsor applied a correction factor of 3.2 ($[40.1 \text{ mg/kg}] / [12.4 \text{ mg/kg}]$) to all total radioactive residue concentrations from Table 10. This correction factor assumes pharmacokinetic linearity over this dose range in pigs, which has not been confirmed but is extrapolated from the results of the toxicokinetic study in dogs (El Amrani-Callens, 2016). The sponsor then derived MR:TRR ratios by combining the marker results of the non-radiolabelled study (Hall, 2017) with the dose-corrected radiolabelled total residue results from McLean, 2016. A second approach for calculating the MR:TRR ratio was considered by the sponsor, using only the marker residue concentrations from McLean, 2016 as assessed by the LC-MS/MS method (Ward, 2017a). However, this approach was considered inappropriate because only two samples per time point were analysed, and some tissue samples were deemed unstable after 440 d storage.

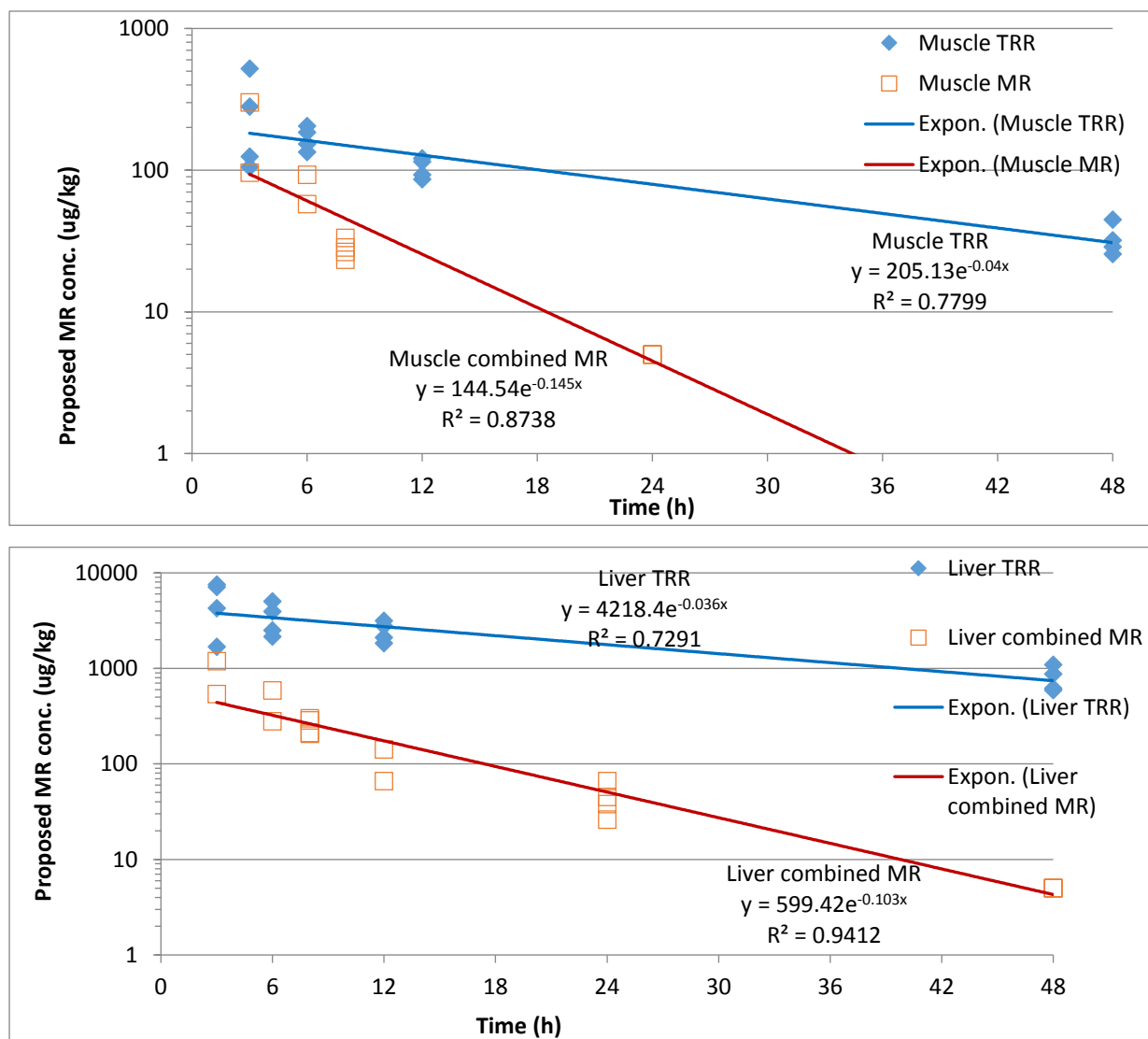
The Committee independently examined the validity of the sponsor's proposed approaches. Preliminary halquinol MR:TRR ratios were further derived using modifications of the proposed approaches, including combining marker residue values at various withdrawal times from both non-radiolabelled (Hall) and radiolabelled (McLean) studies. Note that for purposes of residue depletion modelling, the Committee applied the following criteria to the (non-radiolabelled) marker residue data:

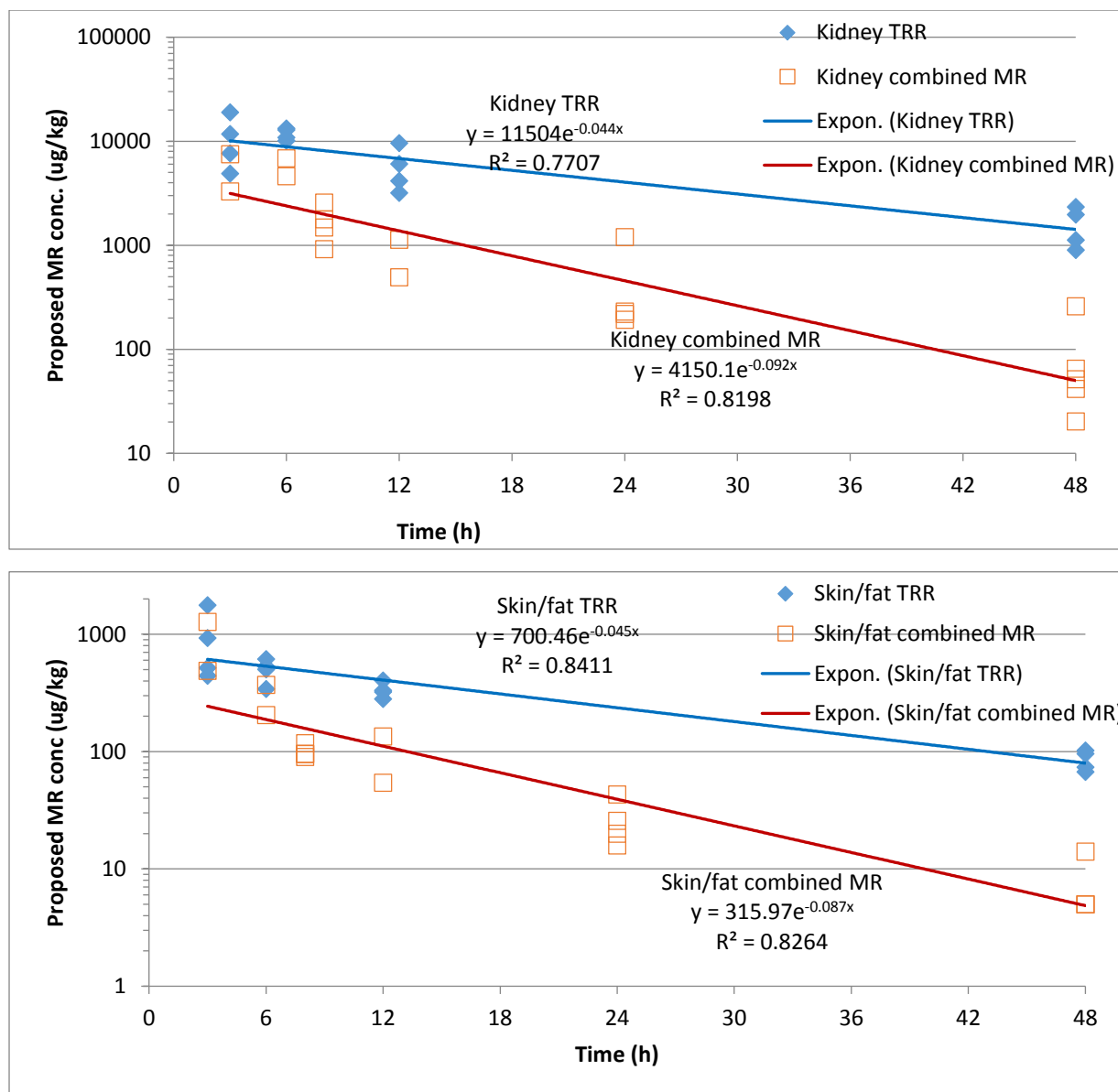
- Numerous tissue samples had one halquinol component (typically 5-CL) that was detectable ($>0.4 - 4 \text{ } \mu\text{g/kg}$, depending on component and tissue) but $<\text{LOQ}$ ($10 \text{ } \mu\text{g/kg}$), while the concentration of the other component in the same sample was $> \text{LOQ}$. In this case a value of $\frac{1}{2} \text{ LOQ}$ ($5 \text{ } \mu\text{g/kg}$) was applied for the $<\text{LOQ}$ component. This was deemed to be more appropriate than excluding the $<\text{LOQ}$ component entirely (assigning a zero value), or using the numeric (but $<\text{LOQ}$) value as reported.
- For tissue samples in which both halquinol components were less than their individual LOQs, the total sample was also considered $<\text{LOQ}$.
- For each tissue, the first withdrawal time with all MR concentrations $<\text{LOQ}$ was included in the residue depletion model and assigned values of $\frac{1}{2} \text{ LOQ}$. However, subsequent time points with all concentrations $<\text{LOQ}$ were not included, so as not to skew the regression.

The log-transformed marker residue concentrations (combined from results of McLean 2016 & Hall 2017 studies) and dose-corrected total radioactive residue concentrations (McLean, 2016) were plotted for each tissue and an exponential regression was performed. From this

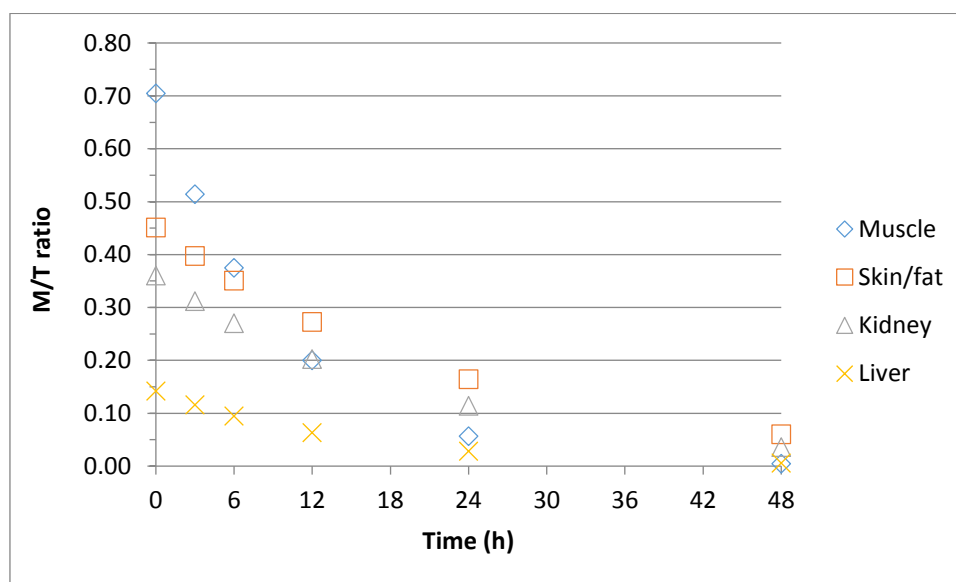
regression, a marker to total ratio was initially determined at 12, 24, 48 and 120 h withdrawal. However, it was readily apparent to the Committee that any regression beyond 48 h was inappropriate, primarily due to the limited numbers of quantifiable MR and TRR concentrations in most tissues beyond 24 – 48 h. Figure 6 shows the MR:TRR regressions for each tissue.

Figure 6. Preliminary MR:TRR regression analysis in swine tissues.





Based on the regression equations derived from Figure 6, preliminary halquinol MR:TRR ratios at various times from 0 – 48 h withdrawal were predicted for each swine tissue (Figure 7).

Figure 7. Predicted halquinol MR:TRR ratios in swine tissue over time.

Methods of analysis for residues in tissues

A validated regulatory method suitable for the routine analysis of halquinol residues in edible swine tissues according to the criteria established by the CCRVDF, as contained in CAC/GL 71-2009, was available. Briefly, the method for the extraction and analysis of halquinol marker residue is as follows: To a ground 1.0 g tissue sample, > 2000 IU glucuronidase/mL in 25 mM sodium acetate, 9 % w/v EDTA, and 100 mM quinidine and diclofenac in acetonitrile is added together with the internal standards for the parent 5,7-DCL, 5-CL, and their sulphate and glucuronide metabolites, and the sample is homogenized in a Genogrinder for 3 minutes. Samples are centrifuged and then incubated in a water bath at 37 °C for between 2 and 2.5 h (Note: skin with fat samples should be re-homogenized and centrifuged after approximately 1 h incubation, and then returned to the incubator for the remaining time). Samples are then extracted with acidified acetone, homogenized again for 3 mins, and additional EDTA is added to the sample and centrifuged for 5 minutes. The supernatant is decanted and retained; the pellet is extracted a second time with acidified acetone. The pelleted sample is further homogenized for 3 mins and then mixed vigorously for 30 minutes. EDTA is added to the extract, centrifuged, and the supernatant is decanted and combined with the retained supernatant from the first extraction. All samples are made up to 10 mL with 20 mM EDTA and centrifuged for 5 minutes. A 500 µL aliquot of the resulting sample is transferred to a 96 well plate or vial and 1 mL of Milli-Q water is added to all samples. The extract is injected onto an equilibrated LC-MS/MS, which is calibrated with injections of varying concentrations of pure standards (1 to 100 µg/mL, equivalent to 10 to 1000 µg/kg, for extracts not requiring further dilution) and analysed for the proposed halquinol marker residue which is defined as the sum of 5-CL, 5,7-DCL, 5-CLG, and 5,7-DCLG using the validated analytical method NAH-16-032 v1.3 (Ward, 2017a) with a LOQ of 10 µg/kg.

A gradient elution is employed on a reverse-phase column, followed by electrospray ionization in positive mode. Detection is by tandem MS, with an analyte specific quantifying transition

and one qualifying transition for confirmation, all of which are monitored simultaneously. Quantification in unknown samples was done by comparing the analyte:IS peak area ratios to those of the calibration curve (except for 5-CL in skin with fat, which was based on peak area) generated by weighted nonlinear (quadratic) regression ($1/x^2$ except $1/x$ for kidney; origin excluded).

Validation of method for residues for bovine tissues

Analytical Procedure NAH-16-032 was validated under GLP compliance (Ward, 2017b). However, the validation was not conducted using the traditional procedure of validating at 0.5, 1 and 2 times the proposed kidney and liver MRLs. No rationale was provided for the non-traditional approach used.

Selectivity instead of Specificity

The detection method (MS/MS) is highly specific for the target analytes. For each tissue type, six sources were tested for interference at LOQ.

In kidney and skin with fat, the interference calculated as the percent peak area of the lowest calibration standard equivalent to the assay LOQ, was less than 2 % for all sources against the analytes (5-CL and 5,7-DCL) as well as the internal standard. In liver, no interference was detected for 5-CL or the internal standard; a single liver source produced an interference equivalent to 5.72 % of the lowest calibration standard for 5,7-DCL. In muscle, one tissue source showed higher interference for both the 5-CL and 5,7-DCL analytes at 9.46 % and 8.51 %, respectively; the internal standard in muscle showed interference of less than 2 %. In all sources, for all matrices and analytes the percent interference did not exceed 10 % of the lowest calibration standard or 5 % of the mean IS peak in a blank sample.

Additionally, several commonly used swine drugs were tested for interference with the chromatographic method, to ensure no chromatographic interference with 5-CL or 5,7-DCL in the presence of other drugs. The compounds tested were fenbendazole, tylosin, tilmicosin, ivermectin, tetracycline, and enrofloxacin. None of the compounds interfered with the performance of the assay.

Accuracy

For a range of fortifications, the mean accuracy was acceptable, falling within -30 to +10 % for samples ≥ 10 to <100 $\mu\text{g/kg}$ and -20 to +10 % for samples ≥ 100 $\mu\text{g/kg}$. In kidney, an additional concentration, at 5000 $\mu\text{g/kg}$ was fortified to demonstrate accuracy following dilution. The results of fortified QC samples generated during the validation are shown in Table 13 and Table 14.

The accuracy results for each tissue (and each analyte) at the 100 $\mu\text{g/kg}$ fortification include one batch ($n=6$) of samples that were hydrolyzed from a fortified sample of glucuronide conjugate.

Precision

Precision under repeatability conditions for the method was acceptable, with inter-day precision of any given matrix and analyte ≥ 10 to <100 $\mu\text{g/kg}$ not exceeding 16.8 % (VICH acceptance criteria is ≤ 23 %) and any given matrix and analyte ≥ 100 $\mu\text{g/kg}$ not exceeding 12.2 % (VICH acceptance criteria is ≤ 16 %).

Intra-day precision for muscle and skin with fat met VICH guidelines for all matrixes, analytes and concentrations (for ≥ 10 to <100 $\mu\text{g/kg}$ % RSD ≤ 15 %; for ≥ 100 $\mu\text{g/kg}$ % RSD ≤ 10 %). The intra-day precision at 500 $\mu\text{g/kg}$ 5,7-DCL in liver and kidney each had one occasion in which the intra-day precision exceeded 10 %. The intra-day precision for 5-CL in liver and kidney each had several occasions and concentrations in which the intra-day precision exceeded VICH recommendation; to account for this, the method recommends that incurred samples should be assayed in duplicate.

Table 13: Accuracy and precision of 5-chloroquinolin-8-ol (5-CL) recoveries in tissues

5-Chloroquinolin-8-ol												
Fortification	Liver			Kidney			Muscle			Skin w/Fat		
$\mu\text{g/kg}$	n	Mean % Accuracy	%CV	n	Mean % Accuracy	%CV	n	Mean % Accuracy	%CV	n	Mean % Accuracy	%CV
10	18	89.2	10	18	89.4	8.05	18	95.1	9.54	18	98.8	9.76
30	18	98.5	16.4	18	92.8	6.19	18	96.9	5.33	18	93.1	15.9
100	23	98.1	10.3	24	90.3	9.84	22	96.3	6.77	24	97.1	6.55
500	17	100	12.2	18	94.4	11.0	18	98.9	4.55	18	102	5.10
5000 (1:10 dilution)	--	--	--	6	104	5.74	--	--	--	--	--	--

Table 14: Accuracy and precision of 5,7-dichloroquinolin-8-ol (5,7-DCL) recoveries in tissues

5,7-Dichloroquinolin-8-ol												
Fortification	Liver			Kidney			Muscle			Skin w/Fat		
$\mu\text{g/kg}$	n	Mean % Accuracy	%CV	n	Mean % Accuracy	%CV	n	Mean % Accuracy	%CV	n	Mean % Accuracy	%CV
10	18	95.4	11.5	18	99.3	9.43	18	106	6.75	18	96.0	12.6
30	18	106	11.1	18	102	6.69	18	107	4.82	18	98.6	11.8
100	23	103	7.67	24	98	8.62	22	107	5.08	24	101	8.65
500	17	106	9.71	18	102	10.7	18	108	7.11	18	103	6.90
5000 (1:10 dilution)	--	--	--	6	104	5.37	--	--	--	--	--	--

Limit of detection and quantitation

The calculated LODs, based on analyses of control samples ($n = 4$ replicates in 6 independent sources), are shown in Table 15. Calculated LOQs, based off the analysis of 6 independent tissues sources, are shown in Table 15. However, accuracy and precision were demonstrated at the nominal LOQ of 10.0 $\mu\text{g/kg}$ in all edible tissues and these were acceptable, therefore the validated LOQ is 10.0 $\mu\text{g/kg}$.

Table 15: Calculated LOD and LOQ

Tissue	n	LOD (µg/kg)		Calculated LOQ (µg/kg)	
		5-CL	5,7-DCL	5-CL	5,7-DCL
Liver	23	0.557	1.14	1.15	2.51
Kidney	24	0.261	2.03	0.535	3.93
Muscle	24	0.211	0.292	0.427	0.577
Skin with Fat	24	0.250	0.373	0.471	0.764

Practicability and applicability under normal laboratory conditions

The method executes in a highly robust manner, but is time consuming due to the hydrolysis and incubation steps. Additionally, to account for the occasional higher variation in precision of the method, it is recommended that incurred samples are assayed in duplicate, to confirm assay results. The method does not utilize any hazardous steps. Up to 20 samples can be processed in a working day and automated injections can be performed overnight.

Table 16: Validated parameters for the LC-MS/MS method for the analysis of the marker residue in edible swine tissues

	5-CL in Liver Tissue	5-CL in Muscle Tissue
	Fortified samples	Fortified samples
Intraday accuracy (% bias)	84.8 - 110	88.8 - 104
Intraday precision (% CV)	2.62 - 19.2	2.17 - 8.74
Interday accuracy	89.2 - 100	95.1 - 98.9
Interday precision	10.0 - 16.3	4.55 - 9.54
LOQ/LOD µg/kg	0.557/1.15	0.211/0.427
Analytical range	10 - 1000	10 - 1000
Specificity/selectivity	No interference observed	No interference observed
Ruggedness testing	Acceptable	Acceptable
Extraction recovery	43.5 - 50.7 %	74.8 - 77.9 %
Stability:		
• Freeze-thaw	3 cycles (-80C)	3 cycles
• Stock solution	78 days at < 20 °C	
Confirmatory analysis:		
• Incurred samples	< 10 %	<10 % with 2 exceptions

	5-CL in Kidney Tissue	5-CL in Skin with Fat Tissue
	Fortified samples	Fortified samples
Intraday accuracy (% bias)	83.3 – 99.0	74.9 - 106
Intraday precision (% CV)	2.93 – 11.8	1.43 – 10.4
Interday accuracy	89.4 – 94.4	93.1 - 102
Interday precision	6.19 – 11.0	5.10 – 15.9
LOQ/LOD µg/kg	0.261/0.535	0.250/0.471
Analytical range	10 - 1000	10 - 1000
Specificity/selectivity	No interference observed	No interference observed
Ruggedness testing	Acceptable	Acceptable
Extraction recovery	87.8 – 90.0 %	85.4 – 87.7 %
Stability:		
• Freeze-thaw	3 cycles	3 cycles
• Stock solution	78 days at < 20 °C	
Confirmatory analysis:		
• Incurred samples	< 10 %	<10 % with 2 exceptions

	5,7-DCL in Liver Tissue	5,7-DCL in Muscle Tissue
	Fortified samples	Fortified samples
Intraday accuracy (% bias)	89.9 - 116	88.8 - 104
Intraday precision (% CV)	4.02 – 13.7	2.17 – 8.74
Interday accuracy	95.4 - 106	95.1 – 98.9
Interday precision	7.67 – 11.5	4.55 – 9.54
LOQ/LOD µg/kg	1.14/2.51	0.292/0.577
Analytical range	10 - 1000	10 - 1000
Specificity/selectivity	No interference observed	No interference observed
Ruggedness testing	Acceptable	Acceptable
Extraction recovery	55.0 – 59.8 %	74.2 – 75.9 %
Stability:		
• Freeze-thaw	3 cycles	3 cycles
• Stock solution	78 days at < 20 °C	
Confirmatory analysis:		
• Incurred samples	< 10 %	<10 % with 2 exceptions

	5,7-DCL in Kidney Tissue	5,7-DCL in Skin with Fat Tissue
	Fortified samples	Fortified samples
Intraday accuracy (% bias)	91.2 – 106	89.8 - 106
Intraday precision (% CV)	3.65 – 15.7	3.33 – 12.8
Interday accuracy	98.0 – 102	96.0 - 103
Interday precision	6.69 – 10.7	6.90 – 12.6
LOQ/LOD µg/kg	2.03/3.93	0.373/0.764
Analytical range	10 - 1000	10 - 1000
Specificity/selectivity	No interference observed	No interference observed
Ruggedness testing	Acceptable	Acceptable
Extraction recovery	85.8 – 86.8 %	68.5 – 69.6 %
Stability:		
• Freeze-thaw	3 cycles	3 cycles
• Stock solution	78 days at < 20 °C	
Confirmatory analysis:		
• Incurred samples	< 10 %	<10 % with 2 exceptions

Stability of residues

Stock and working solutions of parent analytes (5-CL and 5,7-DCL) are prepared in ACN and should be stored at <20°C in polypropylene storage containers. Stock solutions of parent analytes and the internal standard are stable for at least 78 days and working solutions of the parent analytes are stable for at least 24 days. Internal standard working solution is prepared fresh daily.

Stock and working solutions of glucuronide standards were tested for stability at 4°C and -20°C. Stock and working solutions are prepared in methanol. Stock and working solutions of glucuronide standards have been shown to have < -8 % difference from freshly prepared standards at 4°C after 63 days. It is recommended that solutions be stored at -20°C.

Incurred sample stability was evaluated in the residue depletion study (Hall, 2017). Incurred samples in muscle, liver, kidney and skin and fat were evaluated for stability at 34, 36, 35 and 35 days respectively. All samples analyzed for stability (except for the 36 day liver stability time point from a single animal) were within 88-103 % of the initial value. The liver stability time point was 64.8 % of the initial value; no assignable cause could be determined for the low result. Fortified sample frozen storage stability at -80°C has been confirmed for 5-CL and 5,7-DCL up to the following durations. Additional stability storage stability analysis were conducted for 31 days in kidney, 22 days in liver, 33 days in muscle and 61 days in skin with fat.

All matrices and analytes confirmed bench top stability for at least 2 h at ambient temperatures; the percent difference from baseline following 2 h storage did not exceed more than 17.7 % in any tissue or analyte (liver, 30 µg/kg, 5-CL).

All matrices and analytes confirmed freeze/thaw stability up to three cycles; the percent difference from the freshly prepared QCs and the baseline samples following 3 freeze/thaw cycles did not exceed -16.0 % in any tissue or analyte (skin with fat, 500 µg/kg, 5,7-DCL).

Therefore, stability of samples was adequately demonstrated for normal conditions of laboratory handling.

Appraisal

Because of the significant differences in halquinol dose ranges used, methods of oral administration, plasma sampling, schedules and assay characteristics (LLOQ) for each pharmacokinetic study, it is difficult to compare halquinol pharmacokinetics between species. Direct comparison of C_{max} and AUC is not appropriate. 5-CL concentrations were non-detectable in all but one plasma sample from all PK studies combined. 5,7-DCL was detectable after oral halquinol administration in all species tested, with rapidly declining plasma concentrations. Significant bioaccumulation after repeated dosing was not observed in any species, and no significant gender effect was noted on halquinol pharmacokinetics in any species. For 5-CL, the glucuronide metabolism predominates in the rat and pig, whereas the sulfate pathway predominates in the dog. For 5,7-DCL, only the glucuronide pathway produced quantifiable metabolites in the pig. However, the sulfate pathway predominates for 5,7-DCL in the dog, and both pathways produce comparable concentrations in the rat. A comparative metabolism study demonstrated qualitatively similar metabolic profiles of 5,7-DCL in Sprague Dawley rats, dogs, minipigs, pigs and humans, though it is noted that this *in vitro* study did not identify sulfate metabolites of 5,7-DCL in dogs or rat hepatocytes or microsomes (though 5,7-DCLG was produced by these species *in vivo*).

Radiolabelled halquinol residue depletion studies conducted in swine show that halquinol is rapidly metabolized and excreted in the urine and feces within 48 h of the last dose administered. Extractability of radiolabelled halquinol residues was much greater in muscle and skin with fat than in liver and kidney. The extractability was lowest in liver (≤ 50 % for all samples assessed) and highly variable in kidney. One potential explanation for the poor extractability in these samples may be protein binding of residues. The lack of extractability of radiolabelled residues in liver and kidney decreases the confidence of the derived total radioactive residues (TRR) in these tissues. Furthermore, the lack of characterization of these non-extractable residues, as well as many of the extractable (but not defined) metabolites present on the liver and kidney chromatograms, is problematic.

The halquinol marker residue was proposed by the sponsor to be the sum of 5-CL, 5,7-DCL, 5-CLG (expressed as 5-CL equivalents), and 5,7-DCLG (expressed as 5,7-DCL equivalents).

Multiple approaches were used by the Committee to determine the halquinol marker residue (based on the sponsor's proposed MR) to total residue ratios for each edible tissue. In an effort

to characterize the changing MR:TRR over time, a combination of two separate study results (MR results from Hall 2017, TRR results from McLean 2016) was considered. This was based on a preliminary proposal by the sponsor using the lower bound of estimated MR:TRR ratios, which was acknowledged to be more conservative (leading to higher predicted total residues). However, the Committee considers it inappropriate to predict total residues based on potentially unsound MR:TRR estimates, and these approaches used to derive MR:TRR ratios were ultimately deemed unsuitable due to the following reasons:

- A greater than 3-fold difference in halquinol doses used between the studies (acknowledging that while the pharmacokinetics of halquinol may be linear over this dose range in other species, this has not been demonstrated conclusively in pigs);
- The discordance between the halquinol MR:TRR ratios derived from the radiolabelled study alone, compared to the regression approach derived from the combination of radiolabelled and non-radiolabelled data;
- The generally low amount of total and/or extractable radioactivity observed in swine tissues may cause unacceptable uncertainty in the MR and TRR counts;

Furthermore, due to insufficient characterization of the total metabolite profile in edible tissues (particularly liver and kidney), the Committee cannot confirm the suitability of the sponsor-proposed marker residue at this time. Without further characterisation of the unknown non-extractable halquinol residues, and the undefined extractable metabolites, the proposed halquinol marker residue may not be appropriate. Therefore any MR:TRR ratio determined at this time could only be a preliminary estimate, subject to further assessment once an appropriate marker residue has been confirmed.

The non-radiolabelled halquinol residue depletion data (Hall, 2017) were sufficient to determine median sponsor-proposed marker residues and subsequent 95/95 UTLs in muscle, liver, kidney, and skin with fat for time periods up to 120 h post-withdrawal (data not shown). However, the total residue of concern cannot be estimated with confidence from these (proposed) marker residue concentrations due to insufficient MR:TRR data. As the residue of concern may be based on total halquinol residues, and not any specific residue components (e.g. 5-CL, 5,7-DCLG and their glucuronide metabolites), accurate prediction of total halquinol residues from robust MR:TRR ratios is essential.

An LC-MS/MS method has been developed and validated for the radiolabelled and proposed marker residue depletion studies in swine. The LOQ of the method is 10 µg/kg. The stability of samples was adequately demonstrated for normal conditions of laboratory handling.

MRLs could not be recommended for halquinol due to the lack of an established HBGV, incomplete characterization of residues in tissues (particularly liver and kidney), and a lack of data necessary to establish reliable MR: TRR ratios over time for calculation of total residues in tissues. The suitability of the proposed marker residue for halquinol cannot be confirmed without further characterisation of the residues.

Dietary Exposure Assessment

Dietary exposure to halquinol may occur only through its use as a veterinary drug. There is no registered use for halquinol as a pesticide. The Committee has not previously considered halquinol as a veterinary drug.

No dietary exposure assessments were performed for halquinol in swine tissues due to the lack of residue characterization, total residue concentrations, and established health-based guidance value.

Maximum Residue Limits

In considering MRLs for halquinol in swine, the committee considered the following factors:

- A microbiological ADI of 0–0.3 mg/kg bw and a microbiological ARfD of 0.9 mg/kg bw were established by the Committee. A toxicological ADI could not be established due to the lack of information required to assess the *in vivo* mutagenicity and carcinogenicity potential of halquinol. It was not possible to establish an ADI for halquinol in the absence of a toxicological ADI.
- Withdrawal periods range from 0 to 7 days for approved veterinary uses in swine.
- Halquinol is extensively metabolized in swine, primarily to glucuronide metabolites.
- The Committee cannot confirm the suitability of the sponsor-proposed marker residue at this time due to insufficient characterization of the total metabolite profile in edible tissues.
- In the absence of an acceptable marker residue, the Committee cannot comment on the suitability of any analytical method for halquinol residue monitoring purposes.
- The non-radiolabelled halquinol residue depletion data were sufficient to determine median sponsor-proposed marker residues and 95/95 UTLs in muscle, liver, kidney, and skin with fat for time periods up to 120 h post-withdrawal. However, the total residue of concern cannot be estimated with confidence from these proposed marker residue concentrations due to insufficient MR:TRR data. The residue of concern is likely to be total halquinol residues and not any specific residue components.

MRLs could not be recommended for halquinol due to the lack of an established HBGV, incomplete characterization of residues in tissues (particularly liver and kidney), and a lack of data necessary to establish reliable MR:TRR ratios over time for calculation of total residues in tissues.

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Ward, C. 2017b. Validation of analytical method NAH-16-032 (Version 1.0) for the determination of residues of halquinol in porcine muscle, liver, kidney and skin with fat by LC-MS/MS. Study Number NAH-16-032. Sponsor submitted.

Lufenuron

First draft prepared by

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Identity

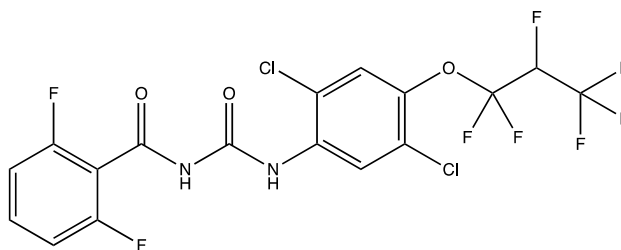
International Non-proprietary Name (INN): Lufenuron

Synonyms: IMVIXATM, Match EC, Match 5 EC, Curyom 550 EC

IUPAC Name: (RS)-1-[2,5-dichloro-4-(1,1,2,3,3,3-hexafluoro-propoxy)-phenyl]-3-(2,6-difluorobenzoyl)-urea

Chemical abstract service N°: 103055-07-8

Structural formula:



Molecular formula: C₁₇H₈Cl₂F₈N₂O₃

Molecular weight: 511.15 g mol⁻¹

Other information on identity and properties

Pure active ingredient: Lufenuron (purity ≥ 98 %)

Appearance: White to pale yellow powder

Melting point: 174.1 °C

Solubility in water: 46 µg/L at 25 °C

Solubility in methanol: 45 g/L at 20 °C

Solubility in acetonitrile: 50 g/L at 20 °C

Solubility in dichloromethane: 70 g/L at 20 °C

Vapor pressure: <1.3 x 10⁻⁷ Pa at 20 °C

Log K_{o/w}: 5.12

Chirality: Equimolar racemic mixture of R- and S-isomers

Polimorphism: Seven crystalline forms

Impurities: Less than 2 % (total) and less than 0.5 % for each compound. 2,6-difluoro benzamide, N-(2,5-dichloro-4-hydroxyphenyl-aminocarbonyl)-2,6-difluoro-benzamide, N-[5-chloro-4-(1,1,2,3,3,3-hexafluoropropoxy)-phenyl-aminocarbonyl]-2,6-difluoro-benzamide, N-[2-chloro-4-(1,1,2,3,3,3-hexafluoropropoxy)-phenyl-aminocarbonyl]-2,6-difluoro-benzamide, N-[2,5-dichloro-4-(1,1,2,3,3,3-hexafluoropropoxy)-phenyl-aminocarbonyl]-2-chloro-6-fluoro-benzamide, N-[2,5-dichloro-4-(1,1,2,3,3,3-hexafluoropropoxy)-phenylaminocarbonyl]-2-fluoro-benzamide, N,N'-bis[2,5-dichloro-4-(1,1,2,3,3,3-hexafluoropropoxy)-phenyl]-urea and carbonic acid 2,5-dichloro-4-[3-(2,6-difluoro-benzoyl)-ureido]-phenylester phenyl ester

Background

Lufenuron (CAS No. 103055-07-8) is a benzoyl phenylurea insecticide used as veterinary drug for the treatment of sea lice infestations (*Caligus rogercresseyi*) in farmed Atlantic salmon (*Salmo salar* L.) and as an insecticide for the control of flea infestation in companion animals (cats and dogs). It is also used in crop protection as an insecticide for various fruits and vegetables, being active against larvae of Lepidoptera and Coleoptera. Lufenuron consists of an equimolar racemic mixture of R- and S- enantiomers.

The toxicology and residues of lufenuron were evaluated by the FAO/WHO JMPR in 2015. At that meeting, JMPR established an ADI of 0-0.02 mg/kg 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. The Committee on this Meeting also concluded that it was not necessary to establish an acute reference dose (ARfD) 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 occasioned by a single dose (JMPR, 2015a).

The U.S. Food and Drug Administration established an import tolerance of 1.35 ppm lufenuron in salmonids muscle with adhering skin (U.S.FDA, 2016).

The European Commission adopted a MRL of 1350 µg/kg lufenuron in fin fish (Regulation EU 967/2014), based on a toxicological ADI of 0.015 mg/kg bw based on the NOEL of 1.5 mg/kg bw/day for the observed signs of hepatotoxicity in a 1-year dog toxicity study (EMA, 2015).

The European Food Safety Authority reviewed the MRL for lufenuron in 2016 (EFSA, 2017).

At the Twentieth Third Session, the Codex Committee on Residues of Veterinary Drugs in Food (CCRVDF) requested evaluation of lufenuron with establishment of MRL in finfish (salmon/trout) muscle and skin in natural proportions.

Residues in food and their evaluation

Conditions of use

Lufenuron is a benzoyl phenyl-urea insecticide used in aquaculture for the treatment of sea lice on farmed salmon. It is also used as an insecticide on a wide range of vegetable crops, oilseeds, root crops maize, sugarcane and coffee for the control of the larvae of many insect pest.

Lufenuron acts by inhibition of chitin synthesis, disrupting the formation of chitin and prevents larvae from moulting.

The treatment is based on administration of a high dose of the active ingredient to Atlantic salmon when they still are in the life stage of smolts and maintained in fresh water. The drug protect fish whilst they are in seawater, facing continual sea-lice pressure.

Dosage

Lufenuron is registered for use in aquaculture in Chile since 2016 with a withdrawal period of 2050 degree-days for salmon. The MRL adopted is 1350 µg/kg of lufenuron in muscle and skin in natural proportions.

The pre-mix containing lufenuron 10 % (w/w), corn starch 88 % (w/w) and colloidal silicon dioxide 2 % (w/w) is incorporated by top-coating or vacuum coating onto fish feed. Considering a feeding rate of 1 % biomass/day the premix incorporation rate would be 5 kg to 1000 kg feed, which represents a final concentration of 0.05 % w/w of lufenuron in the feed.

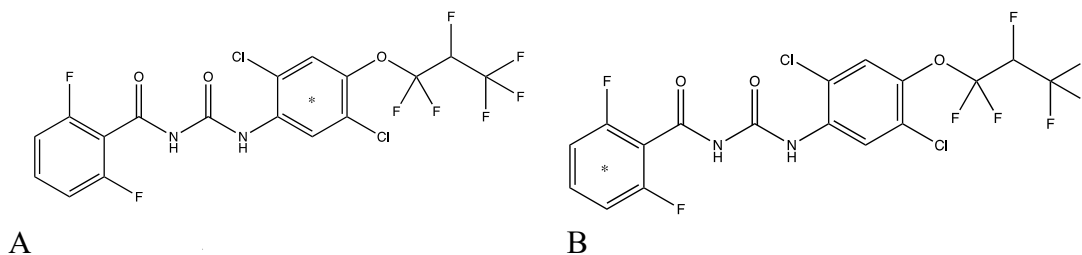
The recommended dose is 5 mg lufenuron per kg of fish biomass orally once daily for seven consecutive days. The treatment starts at the life stage of smolts, when the fish are maintained in fresh water and before they are transferred to sea. When the expected feeding rate is disrupted, the feeding period may need to be extended from 7 days to a maximum of 14 days to ensure the fish receive the full therapeutic dose of 35 mg/kg bw per day.

Pharmacokinetics and metabolism

Test material used in the radiolabelled pharmacokinetic and metabolism studies

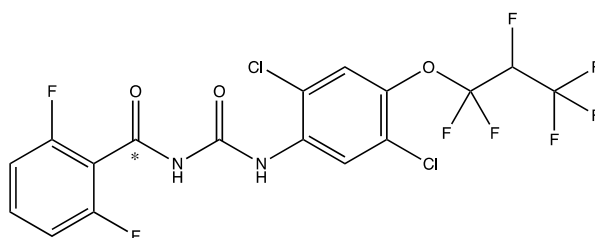
Pharmacokinetic and metabolism studies were conducted with [¹⁴C]-lufenuron labelled within the dichlorophenyl ring (Figure 1A) or the difluorophenyl ring (Figure 1B). The purities of these radiolabelled compounds, determined by high performance liquid chromatography (HPLC), used throughout the studies, were greater than 96 %.

Figure 1. Structure of the radiolabelled compounds: A – [^{14}C]-lufenuron labelled at the dichlorophenyl ring and B - [^{14}C]-lufenuron labelled at the difluorophenyl ring



For the ADME studies on salmon [^{14}C]-lufenuron labelled at the carbon on the benzamide group was used (Figure 2). The purity of the radiolabelled compound, determined by HPLC, was greater than 99 %.

Figure 2. Structure of the [^{14}C]-lufenuron labelled at the carbon of the benzamide group



Pharmacokinetics in laboratory animals

Rats

In a GLP-compliant ADME study (Bissig, 1990), three male and three female rats (Tif: RAIf, SPF), 7 to 11 weeks old and with a body weight of 200-260 g, separated in two groups (E1 and E2), were treated with radiolabelled [^{14}C]-lufenuron (purity higher than 99 %, specific activity 51.4 $\mu\text{Ci}/\text{mg}$, labelled at the dichlorophenyl ring), at two dose levels: 0.5 mg/kg bw and 100 mg/kg bw. Blood was collected at different time points: 1, 2, 4, 8, 12, 24, 32, 48 and 56 h post-dose for Group E1 and 1, 2, 4, 8, 12, 24, 32, 48, 56 h and 72 h post-dose for Group E2. The blood kinetics indicated moderate absorption rates from the intestinal tract into the systemic circulation. The maximum blood levels were reached about 8 h after administration and declined to one half of the maximum value about 45 and 60 h following treatment for the low and high dose level, respectively.

The depletion of residues in tissues followed biphasic first order kinetics with half-life times of 2 to 6 days for Phase 1. For the slower Phase 2, different half-life times were observed for the two dose levels: 5 to 9 days at the low dose level and 10 to 37 days at the high dose level, respectively (Bissig, 1990).

In another study, non-GLP compliant (Okada, 1997), female (3) and male (3) rats (Sprague-Dawley [Crj:CD(SD)]) were treated by oral gavage with radiolabelled [^{14}C]-lufenuron (purity higher than 97.8 %, specific activity 1.89 MBq/mg, labelled at the dichlorophenyl ring) at the

dose of 0.5 mg/kg bw for 14 consecutive days. Blood samples were collected at 8 and 24 h after each daily administration for 13 consecutive days and then 8, 24, 48 and 168 h after the final administration. After the first dose blood levels reached 0.040 µg eq/mL and 0.025 µg eq/mL at 8 h and 24 h, respectively. Lufenuron blood concentrations increased during the dosing period, reaching 0.184 µg eq/mL and 0.178 µg eq/mL in males and females 8 h after the last dose (Day 14). After dosing ceased, lufenuron blood concentrations decreased gradually, reaching 0.095 µg eq/mL and 0.101 µg eq/mL in males and females, respectively, with estimate elimination half-lives of 208 and 323 h in males and females, respectively.

Dogs

Pharmacokinetics and bioavailability of lufenuron in dogs were investigated in two GLP-compliant studies (Maurer and Hotz, 1999). In the first study, two Beagle dogs (Animal 511, female, 15.6 kg and Animal 534, male, 12.3 kg) were treated intravenously with a single dose of 2.6 mg/kg bw [14 C]-lufenuron (purity higher than 99 %, specific activity 51.4 µCi/mg, labelled at the dichlorophenyl ring). The drug was dissolved in Tween 80/Solketal and administered before feeding. For the second study, two Beagle dogs (Animal 508, female, 12.3 kg and Animal 313, male 12.6 kg) were administered a single oral dose of 10 mg/kg bw [14 C]-lufenuron (purity higher than 98.9 %, specific activity 0.104 MBq/mg, labelled at the dichlorophenyl ring). Blood samples were taken at regular intervals for up to 21 days and lufenuron quantified by HPLC. The blood concentration profiles for both studies are shown in Figure 3.

Figure 3. Blood concentration profile of [14 C]-lufenuron following intravenous (IV) and oral administration to Beagle dogs (Maurer and Hotz, 1999). The pharmacokinetic parameters were estimated from both studies and data are summarized in Table 1

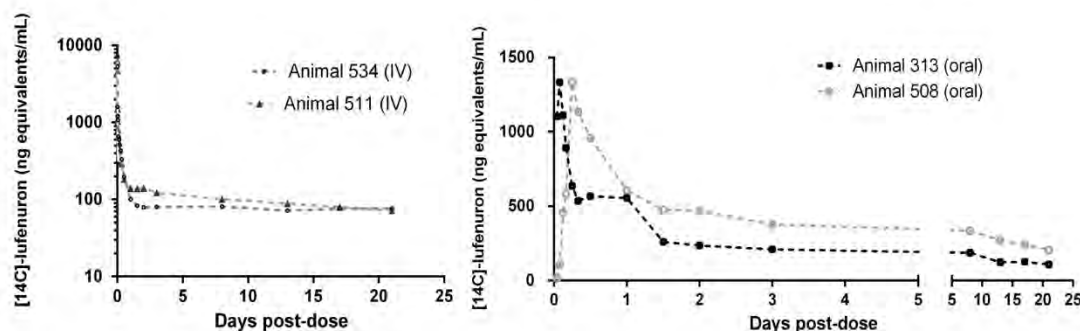


Table 1. Pharmacokinetic parameters for lufenuron in Beagle dog plasma after dosing by intravenous injection or oral administration (Maurer and Hotz, 1999)

Administration route and dose	Dog n.	t _{max} (days)	C _{max} (ng/mL)	t _{1/2} (days)	k _e days ⁻¹	AUC _(0-t) (ng.days/mL)	AUC _(0-inf) (ng.days/mL)	MRT (days)
Oral	508	0.25	1334	20.2	0.034	6920	12890	27.8
10 mg/kg bw	313	0.083	1334	18.0	0.039	3833	6735	24.1
Intravenous	511	0	6223	23.8	0.029	2272	4747	32.9
2.5 mg/kg bw	534	0	145	198.4	0.0035	1905	23682	283.5

t_{max}: time to peak plasma concentration; C_{max}: peak plasma concentration; AUC: area under the curve; t_{1/2}: elimination half-life and MRT: mean residence time.

Animal 534 showed an extremely slow elimination and was considered as an outlier. The bioavailability after oral administration was estimated using AUC_(0-t), instead of AUC_(0-inf) once to the prolonged distribution phase of Animal 534 that overlapped the elimination and thus led to an overestimated elimination half-life time. The bioavailability is estimated to be about 70 %. Due to generally slow elimination of lufenuron a significant amount of the administered dose remained in the bodies of the dogs 21 days after oral administration.

Pharmacokinetic in Food-producing Animals

Salmon

In six GLP-compliant studies, carried out in Canada (3 studies), Chile (one study) and Norway (2 studies), Atlantic salmon smolts (mixed sex) held in a fresh water hatchery were treated with lufenuron at a nominal dose of 5 mg/kg bw for seven consecutive days (Table 2). Fish were transferred to sea cages after the end of medication and remained at sea until the end of the study. Blood samples were collected up to 12 months after the last dose and lufenuron quantified by a validated liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) method, using ¹³C₆-lufenuron as an internal standard. The limit of quantitation (LOQ) of the method was 10 ng/mL. The lufenuron blood depletion curves are shown in Figure 4. Values lower than the LOQ were not used for the calculations of the average values.

The half-life of lufenuron in blood determined in the study carried out in Chile was 53.7 days or 635.1 degree-days. Two additional similar studies were carried out using a lower dose (0.5 mg/kg bw) and a higher dose (10 mg/kg bw) as in the first study. The half-lives were 344 degree-days (23.4 days) and 373 degree-days (25.2 days) for the lower and higher doses, respectively. Some residue data were excluded.

Table 2. Experimental designs used for the determination of lufenuron in blood of Atlantic salmon treated with a nominal dose of 5 mg/kg bw for seven consecutive days

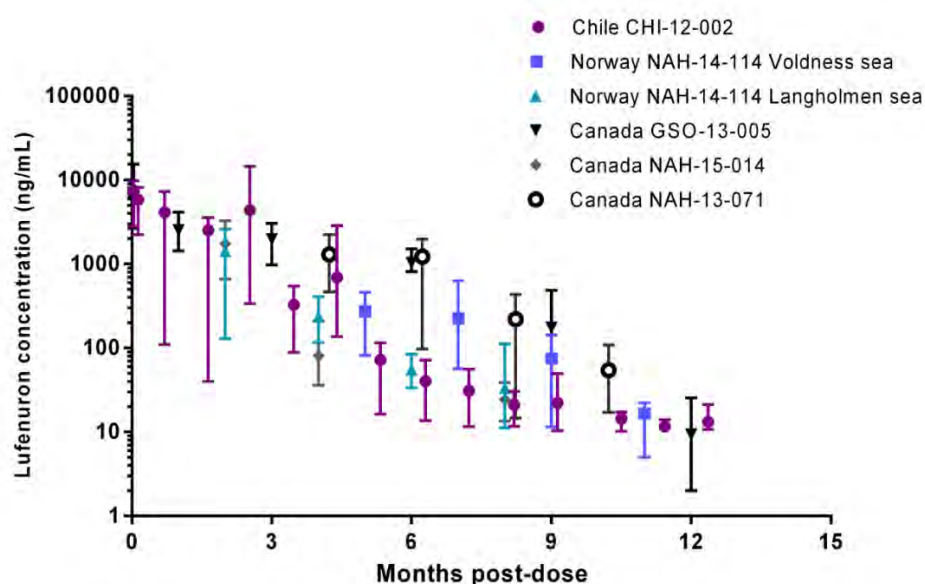
Local of the study	Study number/ Study report	Number of fish	Average weight	Transfer to sea cages after medication	Temperature (°C)		Reference
					Hatchery	Sea	
Canada ^a	GSO-13-005/ ATL-14-1123	36	115 g	9 – 10 days	10.3	1.7- 13.7	Hervy, 2015b
Canada ^b	NAH-13-071/ ATL-15-1351	48	88 g	7 days	10	0.7- 13.2	Hervy, 2016b
Canada ^c	NAH-15-014/ ATL-15-1430	196	62 g	14 days	13	4-16	Hervy, 2016c
Chile ^d	CHI-12-002	124	125 g	6 days	11	9-18	Lewis, 2015
Norway ^e Voldness sea	NAH-14-114/ ATL-15-1352	48	102 g	7 days	10-12	5.8- 15.6	Hervy, 2015d
Norway ^f Langholm en sea	NAH-14-114/ ATL-15-1363	48	98 g	1-3 weeks	3.5	3.7- 13.3	Hervy, 2016a

^aBlood samples were collected at 1 day, 1, 3, 6, 9 and 12 months after the end of the treatment. Six fish were sampled for each time point. ^bBlood samples were collected at 127, 183, 246 and 308 days after the end of the treatment. Twelve fish were sampled for each time point.

^cBlood samples were collected at 2, 4, 6 and 8 months after the end of the treatment. Eighteen fish were sampled for each time point. ^dBlood

samples were collected at 1 (n=6), 4 (n=6), 21 (n=12), 49 (n=11), 76 (n=10), 104 (n=11), 132 (n=10), 160 (n=11), 189 (n=6), 217 (n=9), 246 (n=9), 274 (n=6), 315 (n=4), 343 (n=7) and 371 (n=6) days post last dose.

^eBlood samples were collected at 3, 5, 7, 9 and 11 months after the end of the treatment. Twelve fish were sampled for each time point. ^fBlood samples were collected at 2, 4, 6 and 8 months after the end of the treatment. Twelve fish were sampled for each time point.

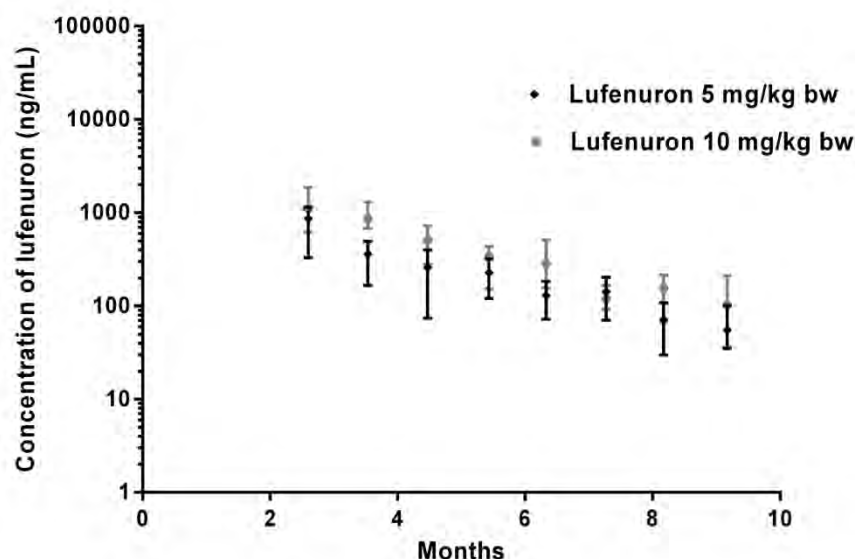
Figure 4. Blood concentration profile of lufenuron following oral administration of 5 mg/kg bw for 7 consecutive days

Rainbow trout

Pharmacokinetics of lufenuron in rainbow trout (*Oncorhynchus mykiss*) were evaluated in a GLP-compliant study conducted in Chile (Lewis and Jacob, 2017). Two groups of rainbow trout smolts (mixed sex and an average weight of 212 g at the beginning of the treatment) held in a fresh water hatchery (11.2 °C) were treated with lufenuron incorporated onto feed at a nominal dose of 5 mg/kg bw and 10 mg/kg bw for seven consecutive days. Fish were transferred to sea cages after the end of medication and remained at sea until the end of the study. The temperature of the sea varied from 9.2 to 17.1 °C. Blood samples (1 mL) drawn from the caudal vein were collected at 1, 6, 21, 49, 78, 106, 134, 163, 190, 218, 245 and 275 days post last dose. Six fish per time point were sampled. In order to provide a reliable depletion curve for blood residues, the data obtained for days 1, 6, 21 and 49 were excluded. The lufenuron blood depletion curves are shown in Figure 5.

The half-time of lufenuron in blood was 591.1 degree-days or 54.1 days for the first treatment group (dose of 5 mg/kg bw for seven consecutive days) and 584.1 degree-days or 53.4 days for the fish that were treated with 10 mg/kg bw for seven consecutive days.

Figure 5. Blood concentration profile of lufenuron in rainbow trout following oral administration of 5 mg/kg bw and 10 mg/kg bw for 7 consecutive days. Studies carried out in Chile



Metabolism in Laboratory Animals

Rats

In a GLP-compliant ADME study, 66 male and 15 female rats (Tif: RAIf, SPF), separated into eight groups (Table 3), were treated with radiolabelled [¹⁴C]-lufenuron (purity higher than 99 %, specific activity 51.4 µCi/mg, labelled at the dichlorophenyl ring), at two dose levels: 0.5 mg/kg and 100 mg/kg (Bissig, 1990). The test substance was dissolved in ethanol:polyethylene glycol 200 (3:7, v/v). Each animal received the dose in 0.5 mL by stomach tube, except the animals of Group C that received the dose in 0.6 mL. The control

animals (12 rats) received a single dose of the administration vehicle without the test substance. The following tissues and organs were collected from the animals of the Groups B, C, D, F1, F2, F3 and F4: bone, brain, abdominal fat, testes, ovaries, uterus, heart, kidneys, liver, lungs, whole blood, plasma, skeletal muscle, spleen, thymus, thyroid and residual carcass.

Table 3. Experimental design for ADME studies of lufenuron in rats (Bissig, 1990)

Group	Animals	Body weight/age	Dose (mg/kg bw)	Sample collection
B	5 males	200 g/ 7 weeks	0.50 – 0.51	Urine ^a , faeces ^a and various tissues after 7 days post-dose
	5 females	200 g/9 weeks	0.51 – 0.52	
C	5 males	210–260 g/8-weeks	0.49 – 0.53 *	Urine, ^a faeces ^a and various tissues after 7 days post-dose
	5 females	210–260 g/11 weeks	0.54 – 0.58 *	
D	5 males	200 g/ 7 weeks	97.7 – 108.1	Urine ^a , faeces ^a , expired air ^b and various tissues after 7 days
	5 females	200 g/9 weeks	100.3 – 109.3	
E1	3 males	200 g/ 7 weeks	0.47 - 0.51	Blood at different time points (1, 2, 4, 8, 12, 24, 32, 48 and 56 h post-dose)
E2	3 females	200 g/9 weeks	102.9 – 103.5	Blood at different time points (1, 2, 4, 8, 12, 24, 32, 48 56 and 72 h post-dose)
F1, F2	20 males	200 g/ 7 weeks	0.47 – 0.53	Various tissues after 8 h, 45 h, 5 days and 12 days post-dose
F3, F4	20 males	200 g/ 7 weeks	91.6 – 101.0	Various tissues after 8 h, 60 h, 5 days and 12 days post-dose
G	5 males (bile fistulated)	260 g/8 weeks	0.48 – 0.49	Bile ^c , urine ^d and faeces ^d

* Preceded by 14 consecutive daily low doses of non-labelled lufenuron. a: 0-8, 8-24, 24-48, 48-72, 72-96, 96-120, 120-144 and 144-168 h post-dose; b: 0-8 and 8-24 h post-dose; c: 0-0.5, 0.5-1, 1-2, 2-4, 4-8, 8-18, 18-24, 24-42 and 42 – 48 h post-dose; d: 0-24 and 24-24 h post-dose.

The extent of absorption of the orally administered lufenuron from the gastrointestinal tract into the systemic circulation was about 45 % and 10 % of the orally administered lufenuron at the low dose (0.5 mg/kg bw, Groups B and C) and the high dose level (100 mg/kg bw, Group D), respectively. Within the first 24 h an average of 25 % and 70 % of the administered dose were eliminated by the animals of Group B and Group D, respectively. Pre-treatment of the animal by 14 consecutive daily oral doses (0.5 mg/kg bw), lightly accelerated the faecal excretion in male rats (Group C). Within 48 h about 2 %, < 1 % and 52 % of the administered dose of the bile cannulated rats (Group G) were excreted with the bile, urine and faeces, respectively. Within 7 days post-dose the male and female rats of Group B and Group C excreted about 45 % and 56 % of the dose. The high dosed animals (Group D) eliminated more than 80 % of the administered dose, almost exclusively with the faeces. Seven days after the single oral dose of [¹⁴C]-lufenuron 0.5 mg/kg bw, significant residues (40 % of the dose in animals of Group B and 10 % of the dose in animals of Group D) were present in most of the tissues collected, especially in fat (1.91 mg eq/kg in male and 2.40 mg eq/kg in female). Less than 0.01 % of the administered radioactivity was detected in exhaled volatile and carbon dioxide traps. The extent of absorption and faecal excretion was dose dependent. The greatest portion of radioactivity in faeces, determined by thin layer chromatography (TLC), was

unchanged parent drug (77 % to 79 % and 37 % to 48 % of the administered high and low doses, respectively). The total radioactivity in faeces varied in the range of 78 % to 80 % and 43 % to 53 % of the high and low doses, respectively. The faecal metabolite pattern of the bile-duct cannulated rats (Group G) was similar to that of animals of Group B, but with a higher percentage (48 %) of the unchanged parent drug. One metabolite was identified as 3,5-dichloro-2,4-difluorophenyl-urea (maximum of 0.2 % of the administered dose in Group C and 0.5 % to 1.0 % of the administered dose in Groups A and B). The urinary metabolite pattern was not determined because the low radioactivity recovered in the samples (less than 1 % of the administered dose). Almost 80 % of the radioactive compounds were extracted from tissues and 95 % from faeces. Profiling of fat, liver, kidneys, lung and carcass indicated only a single component, the parent compound. Moreover, lufenuron was the major component in faeces, 5 minor metabolites were noted, each of 7 % or less of the TRR. Most of the seven biliary metabolite fractions were more polar than the faecal metabolites. Two metabolites were identified: 1-(2,5-dichloro-4-(1,1,2,3,3,3-hexafluoropropoxy)-phenyl)-urea (0.1 %) and (2,5-dichloro-4-(1,1,2,3,3,3-hexafluoropropoxy)-aniline (< 0.1 %).

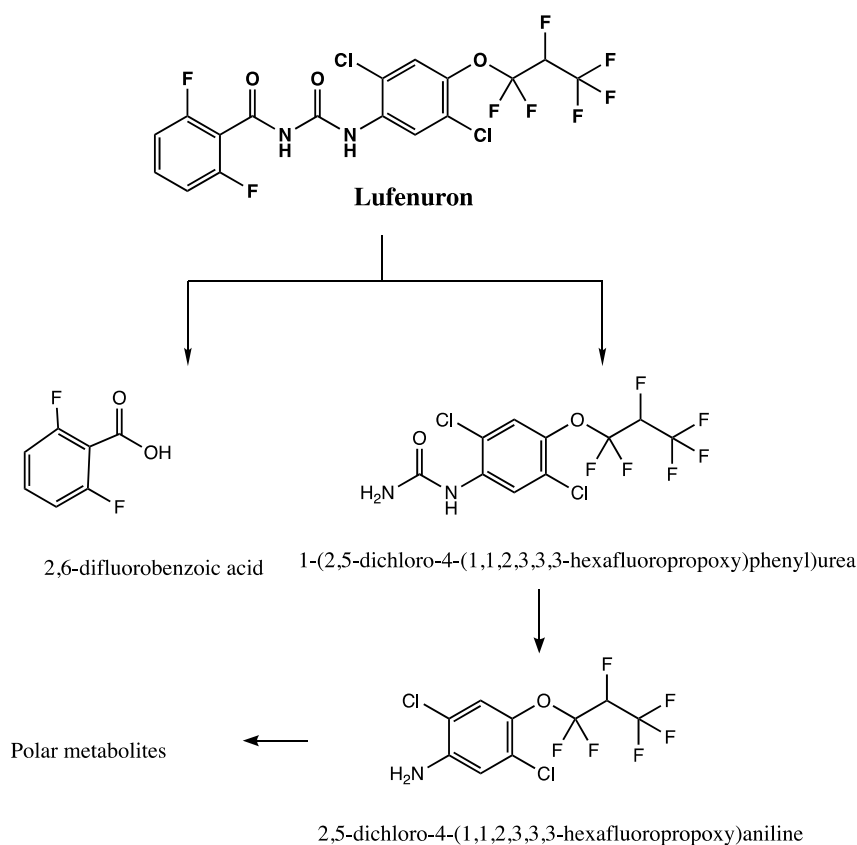
In another study, non-GLP compliant, six weeks old rats of both sexes (Sprague-Dawley [Crj:CD(SD)]) were treated by oral gavage with radiolabelled [^{14}C]-lufenuron (purity higher than 97.8 %, specific activity 1.89 MBq/mg, labelled at the dichlorophenyl ring) at the dose of 0.5 mg/kg bw for 14 consecutive days (Okada, 1997). Whole blood, plasma, cerebrum, cerebellum, pituitary, thyroid, heart, lung, liver, kidney, adrenal glands, spleen, fat, skin, muscle, femur, testis, seminal vesicle, ovary, and carcass were collected and the radioactivity determined by a liquid scintillation counter. Almost 58 to 64 % of the parent compound was excreted until 168 h after the last dose on Day 14. Faecal excretion was the major route of elimination: 57.1 % and 62.1 % of TRR was determined in male and female faeces, respectively. Only 0.9 % and 1.4 % were eliminated into urine from male and female rats, respectively. Concentration in the cerebrum and cerebellum increased with dosing, and were 2-3 times higher than that in blood concentrations by 8 h after the last dose. However, these concentrations were significantly lower than those determined in other tissues (Table 4). At 168 h post-dose, in both sexes, about 1 % of TTR remained in the liver and only 0.1 % or less remained in other tissues. The residual carcasses contained $34.6 \% \pm 4.1 \%$ ($n=3$) and $29.5 \% \pm 1.9 \%$ ($n=3$) of the TTR in males and females, respectively, indicating retention of the non-excreted radiolabelled compound. In plasma, more than 96 % of the radioactivity was extracted with methanol and four radioactive compounds were identified by thin layer chromatography: parent compound (major component, 70-80 %), 1-(2,5-dichloro-4-(1,1,2,3,3,3-hexafluoropropoxy)-phenyl)urea, 2,5-dichloro-4-(1,1,2,3,3,3-hexafluoropropoxy)aniline and an unknown metabolite. In the cerebrum, more than 94 % of the radioactivity was extracted almost in the methanol fraction and a small portion in the n-hexane fraction. Only the parent compound (about 92 %) and 2,5-dichloro-4-(1,1,2,3,3,3-hexafluoropropoxy)aniline (less than 1.1 %) were identified.

Table 4. Average concentration (\pm standard deviation, $n=3$) of radioactivity in rat tissues and organs after 8 h and 168 h post-dose. [^{14}C]-lufenuron was administered orally (gavage) at a dose of 5 mg/kg bw of for 14 consecutive days (Okada, 1997)

Tissue	Concentration of radioactivity ($\mu\text{g eq/g}$ or $\mu\text{g eq/mL}$)			
	Female (8 h)	Female (168 h)	Male (8 h)	Male (168 h)
Blood	0.15 ± 0.01	0.06 ± 0.01	0.15 ± 0.01	0.06 ± 0.01
Plasma	0.18 ± 0.02	0.07 ± 0.00	0.18 ± 0.02	0.07 ± 0.01
Cerebrum	0.38 ± 0.16	0.10 ± 0.02	0.38 ± 0.16	0.10 ± 0.05
Cerebellum	0.35 ± 0.15	0.10 ± 0.103	0.35 ± 0.15	0.11 ± 0.06
Pituitary	1.67 ± 0.27	0.46 ± 0.02	1.67 ± 0.27	0.40 ± 0.01
Thyroid	2.30 ± 0.37	0.53 ± 0.03	2.30 ± 0.37	0.70 ± 0.14
Lung	2.00 ± 0.10	0.73 ± 0.12	2.00 ± 0.10	0.62 ± 0.08
Heart	1.89 ± 0.18	0.63 ± 0.03	1.89 ± 0.18	0.58 ± 0.04
Liver	4.09 ± 0.10	1.26 ± 0.05	4.09 ± 0.10	1.20 ± 0.07
Kidney	2.11 ± 0.12	0.72 ± 0.03	2.11 ± 0.12	0.68 ± 0.04
Adrenal	4.87 ± 0.64	1.62 ± 0.03	4.87 ± 0.64	1.52 ± 0.05
Spleen	1.08 ± 0.04	0.37 ± 0.01	1.08 ± 0.04	0.36 ± 0.05
Muscle	1.02 ± 0.14	0.33 ± 0.02	1.02 ± 0.14	0.35 ± 0.043
Bone	0.41 ± 0.07	0.15 ± 0.02	0.41 ± 0.07	0.15 ± 0.04
Fat	34.68 ± 3.12	18.93 ± 0.53	34.68 ± 3.12	16.59 ± 1.28
Skin	3.74 ± 2.34	2.00 ± 1.2	3.28 ± 0.83	2.14 ± 1.06
Ovary/Testis	2.89 ± 0.27	1.06 ± 0.14	3.74 ± 2.34	0.17 ± 0.05
Seminal vesicle			2.89 ± 0.127	0.722 ± 0.06

In another GLP-compliant study, the nature of the radioactive residues in urine and faeces of male and female rats Tif: RAI f (SPF) treated with seven daily doses of [^{14}C]-dichlorophenyl ring-labelled lufenuron (100 mg/kg bw) was investigated (Thanei, 1990). The radiochemical purity was about 99 % with a specific radioactivity of 51.4 $\mu\text{Ci/mg}$. Faeces were collected at the sampling time 8-24 h and 24-48 h and fat at 168 h post-dose. Almost 100 % of the radioactivity in faeces was extractable with methanol and 97 % with methanol /chloroform in fat. Two non-polar metabolite fractions representing less than 0.5 % of TRR was observed. In this fractions two metabolites were identified by TLC and confirmed by spectroscopic analysis: 1-(2,5-dichloro-4-(1,1,2,3,3,3-hexafluoropropoxy)-phenyl)urea and 2,5-dichloro-4-(1,1,2,3,3,3-hexafluoropropoxy)aniline. Moreover, a cluster of polar metabolites was observed, representing 0.5 % of TRR, however, the compounds remained unidentified.

In summary, lufenuron is mainly excreted in faeces and predominantly retained in fat tissue and therefore poorly metabolized in rats. The minor degradation pathway is the cleavage of the benzamide moiety yielding 1-(2,5-dichloro-4-(1,1,2,3,3,3-hexafluoropropoxy)-phenyl)urea and 2,6-difluorobenzoic acid. Further cleavage of the ureido moiety leads to the aniline derivative 2,5-dichloro-4-(1,1,2,3,3,3-hexafluoropropoxy)aniline (Figure 6).

Figure 6. Proposed biotransformation pathway of lufenuron in rats (Thainey, 1990)

Dogs

In an ADME study two Beagle dogs (one female and one male, 12 – 13 kg body weight) received a single oral dose of 10 mg/kg bw [^{14}C]-lufenuron (purity higher than 98.9 %, specific activity 0.104 MBq/mg, labelled at the dichlorophenyl ring) (Maurer & Hotz, 1999). At the end of the study (21 days post-dose), the animals were sacrificed and the following tissues were collected: skin, brain, liver, subcutaneous fat, kidney fat, omental fat, skeletal muscles, heart, and lungs. The total radioactivity in faeces (days 1 to day 21) was 52 % to 53 % and in urine 0.84 % to 2.18 % of the total administered dose. Fat tissues contributed with 7.2 % to 8.4 % of the TRR. The distribution of radioactivity in tissues and organs are shown in Table 5.

Table 5. Total radioactive residues in tissues and organs after 21 days post-dose. [^{14}C]-lufenuron was administrated orally at a single dose of 10 mg/kg bw (Maurer and Hotz, 1999)

Tissue	TRR (%)	
	Dog n ^o . 1	Dog n ^o . 2
Skin	15.8	4.72
Brain	0.01	0.01
Liver	0.65	0.38
Bile	0.29	0.11
Spleen	0.03	0.01
Fat*	7.21	8.44
Muscle	11.33	6.36
Heart	0.07	0.06
Lungs	0.08	0.04
Kidney	0.05	0.02
Blood	0.3	0.13
Total	35.78	20.28

*Sum of total fat.

The metabolic profiling, using high performance thin layer chromatography (HPTLC), was limited to faecal extracts, once only 0.8 to 2.2 % of the total administered dose was found in urine. At the first day post-dose only the unchanged [^{14}C]-lufenuron was identified in the faeces collected during the period of 0 – 24 h from the two animals. Extracts of the faeces collected between 24 – 48 h post-dose yielded one major peak with an R_f value identical with the reference compound (97.9 %, animal 1 and 64.7 %, animal 2), one minor peak with an R_f higher than the reference compound (less polar compound, 2.1 %, animal 1 and 20.9 %, animal 2) and three other minor metabolites (sum of 14.4 %, animal 2) with very low R_f values (more polar compounds). The identities of the metabolites were not confirmed. In the third fraction of faeces collected over the period of 48 -72 h post-dose, unchanged [^{14}C]-lufenuron was determined at a concentration of 89.9 % (animal 1) and 45.5 % (animal 2). In this fraction, also a peak with a higher R_f value as the parent compound and three minor unidentified peaks (4.8 %, animal 1 and 19.4 %, animal 2) were verified.

Metabolism in Food Producing animals

Goats

In order to study the metabolic profile of lufenuron in goats, [^{14}C]-lufenuron was administered orally (by gavage) for ten consecutive days to two lactating British Sannen goats (Cameron, *et.al.*, 1992a). One goat (40 kg bw) received [^{14}C]-lufenuron labelled in the difluorophenyl ring (specific activity 34.93 $\mu\text{Ci}/\text{mg}$, purity higher than 97 %) at a dose of 8.3-8.5 mg/day and the second goat (38 kg bw) received [^{14}C]-lufenuron labelled in the dichlorophenyl ring (specific activity 51.90 $\mu\text{Ci}/\text{mg}$, purity higher than 98 %) at a dose of 8.5-8.7 mg/day. Following administration of the first dose, urine and faeces were collected in the morning prior to dose administration at 24 h intervals. The sample collection periods were: 0-24 h, 24-48 h, 48-72 h, 72-96 h, 96-120 h, 120-144 h, 144-168 h, 168-192 h, 192-216 h and 216-240 h after the first

dose. Blood samples were collected at 0.5 h, 1 h, 3 h, 6 h, 12 h, 24 h, 48 h, 72 h, 96 h, 120 h, 144 h, 168 h, 192 h and 216 h after the first dose. During the study period the goats were milked in the morning prior to dosing and in the afternoon, ca 7 h after dose administration. The animals were slaughtered 24 h after the last dose and the following tissues were collected: tenderloin muscle, hind quarter muscle, forequarter muscle, omental fat, renal fat, subcutaneous fat, liver, kidneys, rumen and intestinal contents. Radioactivity was measured by combustion and liquid scintillation counting. The main route of elimination of radioactivity was in the faeces, accounting for 73 %-74 % of the total administered dose. Only 1 %-2 % of the total dose was eliminated via urine. The rate of excretion was slow resulting in incomplete excretion of radioactivity over the first 24 h and over the period of the study (240 h). The radioactivity in milk was about 6 %-7 % of the total administered dose over 24 h. The highest level of radioactivity, about 4.2 %-5.4 % of TRR, was determined in fat 24 h post-dose of the tenth day. In muscle, concentration in the range of 0.038 – 0.080 mg eq/kg was detected. Approximately 5 %-7 % of the total administered dose was estimated to remain in tissues at sacrifice and 5 %-10 % remained in the rumen and intestinal contents (Table 6). There were no significant differences in the excretion and distribution pattern of radioactivity observed between the two labelled forms of [^{14}C]-lufenuron.

Table 6. Radioactive tissue residues 24 h following the tenth consecutive daily dose (8.3-8.7 mg) of ([^{14}C]-lufenuron labelled in the difluorophenyl ring or dichlorophenyl ring to a lactating goat (Cameron *et.al.*, 1992a)

Tissue	Goat treated with ([^{14}C]-lufenuron labelled in the difluorophenyl ring		Goat treated with ([^{14}C]-lufenuron labelled in the dichlorophenyl ring	
	Concentration (mg eq/kg)	% Total dose	Concentration (mg eq/kg)	% Total dose
Hindquarter muscle	0.066	1.555*	0.039	0.774*
Forequarter muscle	0.080		0.038	
Tender loin muscle	0.071		0.040	
Omental fat	2.288	5.356*	2.411	4.216*
Subcutaneous fat	0.883		0.821	
Renal fat	2.434		1.640	
Liver	0.417	0.297	0.367	0.281
Kidney	0.114	0.017	0.118	0.014
Blood cells	0.017	0.018	0.015	0.012
Plasma	0.014	0.032	0.017	0.038
Total	-	7.275	-	5.335
Rumen and intestinal contents	0.350	5.043	0.752	10.098

* Calculated on the basis that the total weight of liver, kidney, rumen and intestinal contents is known and that the total weight of muscle and fat represents 45 % of the body weight.

In a separate study, using the samples of the aforementioned study, metabolic profiling was carried out (Schulze-Aurich, 1992). The radioactive residues were almost completely

extractable from all samples (fat, muscle, kidneys, liver, milk, and faeces). In fat, muscle, kidney, and milk unchanged lufenuron was the only detectable residue and accounted for more than 92 % of the extracted radioactivity. In liver besides unchanged lufenuron (>80 %) minor amounts of metabolites were detected. In faeces, besides unchanged lufenuron, 1-(2,5-dichloro-4-(1,1,2,3,3,3-hexafluoropropoxy)phenyl)urea, 2,6-difluorobenzoic acid and 2,6-difluorobenzamide were detected as metabolites. In urine, only 1-(2,5-dichloro-4-(1,1,2,3,3,3-hexafluoropropoxy)phenyl)urea was identified as a minor metabolite.

Chickens

In a GLP-compliant study radiolabelled lufenuron at a dose of 0.59 mg was administered in capsules via pharynx to six laying Leghorn hens daily for 14 consecutive days (Cameron *et.al.*, 1992b). Three animals (1.30 – 1.74 kg body weight) received [¹⁴C]-lufenuron labelled in the difluorophenyl ring (specific activity 34.93 µCi/mg, purity higher than 97 %) and the other three hens (1.35 – 1.59 kg body weight) received [¹⁴C]-lufenuron labelled in the dichlorophenyl ring (specific activity 51.90 µCi/mg, purity higher than 98 %). Radioactivity was measured by combustion and liquid scintillation counting.

Excreta were collected in the morning prior to dose administration at 24 h intervals until 336 h after the first dose. Only a single blood sample was taken at the end of the study prior to sacrifice (336 h after the first dose). Eggs were collected throughout the day and in the morning prior to dose administration over 24 h intervals. At the end of the experiment, 24 h following the fourteenth dose, the animals were slaughtered and the following tissues collected: lean meat, skin, peritoneal fat, liver, kidneys and intestinal contents. Radioactivity was determined using a liquid scintillation counter and the results are presented in Table 7.

Table 7. Tissue concentrations of radioactivity 24 h following the fourteenth consecutive daily dose (0.59 mg) of ([¹⁴C]-lufenuron labelled in the difluorophenyl ring or dichlorophenyl ring to laying hens (Cameron *et.al.*, 1992b)

Tissue	Hens treated with ([¹⁴ C]-lufenuron labelled in the difluorophenyl ring		Hens treated with ([¹⁴ C]-lufenuron labelled in the dichlorophenyl ring	
	Concentration (mg eq/kg)	% Total dose	Concentration (mg eq/kg)	% Total dose
Lean meat	0.24 ± 0.16	1.15 ± 0.61	0.10 ± 0.01	0.55 ± 0.05
Skin (including fat)	2.56 ± 0.51	-	1.30 ± 0.33	-
Peritoneal fat	13.04 ± 0.69	8.83 ± 0.92	7.19 ± 1.32	5.09 ± 1.06
Liver	1.45 ± 0.11	0.64 ± 0.13	0.83 ± 0.14	0.40 ± 0.09
Kidney	0.74 ± 0.03	0.09 ± 0.02	0.52 ± 0.11	0.07 ± 0.03
Whole blood	0.29 ± 0.09	0.14 ± 0.03	0.19 ± 0.31	0.10 ± 0.02
Intestinal contents	0.37 ± 0.06	0.21 ± 0.05	0.41 ± 0.37	0.15 ± 0.03

Recovery of radioactivity was 75 %-79 % of the total administered dose. There were no significant differences in the excretion and tissue distribution pattern dependent on the site of

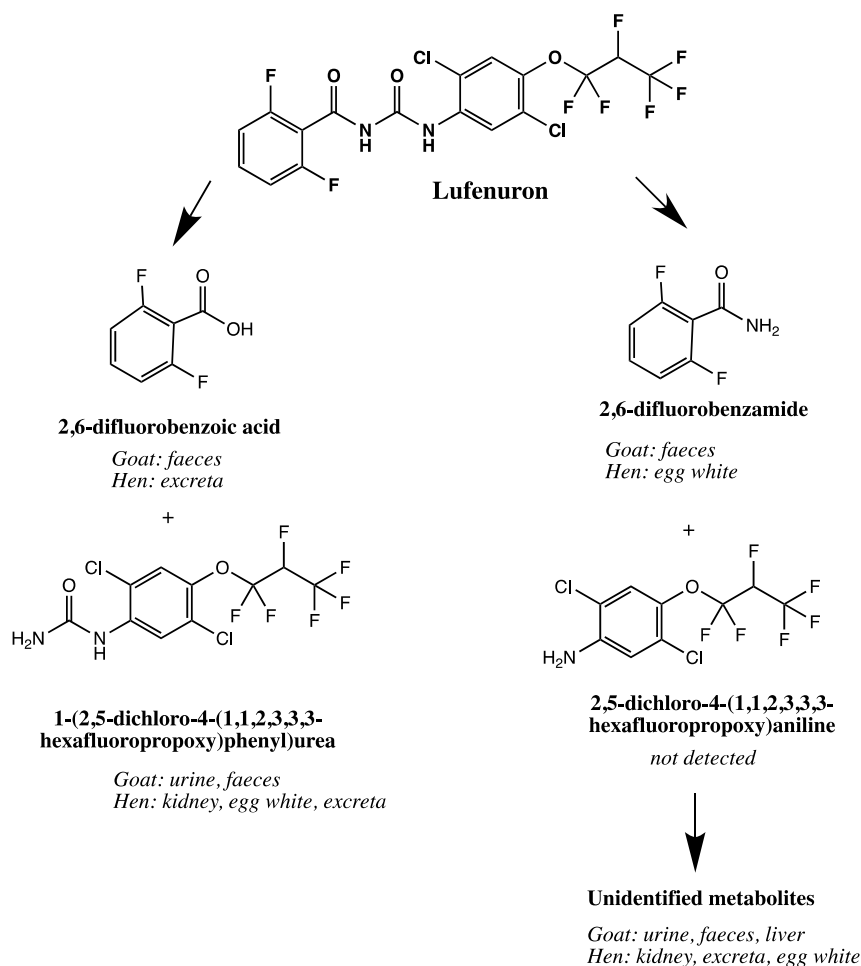
labelling of the test substance. About 10 % of the total dose was eliminated via eggs, mainly in the egg yolk. The majority of the radioactivity (55 %-64 %) was measured in the accumulated excreta. The concentration of radioactivity in peritoneal fat was 38-45 times higher than that in whole blood at the time of sacrifice, indicating selective uptake of the radiolabelled compound and or its metabolites in fat.

The results indicated that the radiolabelled compound is slowly eliminated, mainly by excreta, and tend to accumulate. Highest tissues residues were found in fat.

Metabolic profiling was carried out in another study (Schulze-Aurich, 1992) using the samples of the previous study. Metabolites were characterized by TLC with co-chromatography with standards. The radioactive residues were almost completely extractable with solvents (89 %-98 %) from fat including skin, liver, lean meat, egg white, and egg yolk. For both labels, unchanged lufenuron was the major residue in all tissues and egg yolk (79.3 % – 93.7 % TRR). In egg white, lufenuron summed 37.6 % to 44.1 % of the TRR and 2,6-difluorobenzamide was the only metabolite identified for the difluorophenyl-label (0.001 mg eq/kg, 17.3 % TRR). For the dichlorophenyl-label 1-(2,5-dichloro-4-(1,1,2,3,3,3-hexafluoropropoxy)phenyl)urea was identified in kidney (0.028 mg eq/kg, 5.3 %) and egg white (< 0.001 mg eq/kg, 7% TRR). Unknown metabolites summed 3.1 % to 42.1 % TRR for the difluorophenyl-label and 3.0 % to 37.4 % TRR for the dichlorophenyl label. The highest unidentified metabolites were measured in egg white. In hen excreta > 90 % TRR was extracted and lufenuron was the major compound identified (>82 %). The two minor metabolites detected in kidney and egg white were also identified in excreta, 1-(2,5-dichloro-4-(1,1,2,3,3,3-hexafluoropropoxy)phenyl)urea (3 % TRR) and 2,6-difluorobenzamide (<4.3 % TRR).

The fate of lufenuron in hens and goats after multiple oral administration is assumed to be similar and resembles closely that observed in rats. The same metabolites were identified by co-chromatography in tissues, eggs, milk and excreta and a metabolic pathway of lufenuron in goats and hens was proposed (Figure 7). Lufenuron is metabolized to a limited extent by cleavage of the benzoyl ureido bridge leading to 1-(2,5-dichloro-4-(1,1,2,3,3,3-hexafluoropropoxy)phenyl)urea and 2,6-difluorobenzoic acid and by cleavage of the urea bridge leading to the 2,6-difluorobenzamide and very likely to the 2,5-dichloro-4-(1,1,2,3,3,3-hexafluoropropoxy)aniline (not detected). In goats, the extent of metabolism is assumed to be slightly higher than in hens, as higher amounts of metabolites are eliminated with excreta.

Figure 7. Proposed biotransformation pathway of lufenuron in goats and hens (Schulze-Aurich, 1992)



Salmon

In a GLP-compliant study 64 post-smolt salmon (*Salmo salar* L.) with a body weight of 107-185 g, housed in tanks with seawater, were treated with [^{14}C]-lufenuron (specific activity 95.5 $\mu\text{Ci}/\text{mg}$, purity higher than 99.8 %, labelled at the carbon of the benzamide moiety, Figure 2) at an intended dose of 5 mg/kg bw (calculated dose of 5.34 mg/kg bw) for 7 consecutive days (Hobbs, 2014). The water temperature ranged from 4 to 14 °C. Ten fish were sacrificed at 1 day, 30 days, 90 days and 178 days after the end of treatment and muscle plus skin, liver, hindgut and residual carcasses sampled for analysis (Table 8). Faeces were collected during the treatment period and throughout the live phase. TRR was almost completely extracted from fillet and faeces (>91.2 %).

Table 8. Concentration of total radioactivity in Atlantic salmon tissues with time. Oral administration of [^{14}C]-lufenuron at a dose of 5.34 mg/kg bw for seven consecutive days (Hobbs, 2014)

Time (days)	Concentration of total radioactivity (mg eq/kg) \pm SD			
	Fillet	Liver	Residual carcass	Hindgut*
1	24.8 \pm 7.7	33.9 \pm 9.9	34.8 \pm 9.5	46.9
30	14.3 \pm 8.2	15.8 \pm 8.1	20.1 \pm 14.4	11.1
90	10.5 \pm 5.1	13.2 \pm 5.7	18.1 \pm 8.1	7.3
178	2.7 \pm 1.4	3.5 \pm 1.7	5.3 \pm 2.7	2.2

* Single measurement of combined samples; SD: standard deviation (n= 10 fish).

TRR declined progressively from 1 day after treatment to 178 days post-dose in all tissues. Lufenuron was the only residue detected in all samples analysed accounting for 79.2 % to 95.3 % TRR in the salmon fillet. The concentration of TRR was also determined in blood, organs, and tissues of salmon (mean of 3 fish) at Day 35, Day 90 and Day 178 after the end of the treatment (Table 9).

Table 9. Mean concentration of total radioactivity in Atlantic salmon tissues with time. Oral administration of [^{14}C]-lufenuron at a dose of 5.34 mg/kg bw for seven consecutive days (Hobbs, 2014)

Tissue	Concentration of total radioactivity (mg eq/kg) \pm SD		
	Day 35	Day 90	Day 178
Bladder	4.8 \pm 2.9	3.8 \pm 0.8	0.8 \pm 0.6
Blood	5.7 \pm 1.9	2.8 \pm 0.4	0.6 \pm 0.2
Brain	5.6 \pm 2.9	3.4 \pm 0.6	0.5 \pm 0.3
Brown fat	16.6 \pm 6.2	11.9 \pm 4.2	1.9 \pm 0.8
Muscle	3.4 \pm 1.3	1.8 \pm 0.3	0.3 \pm 0.2
Gill-arch	53.8 \pm 20.6	44.4 \pm 4.6	9.5 \pm 4.2
Gill-rakes	5.0 \pm 2.2	3.9 \pm 0.2	0.7 \pm 0.4
Gill-whole	12.1 \pm 5.4	14.1 \pm 3.2	2.0 \pm 1.0
Heart muscle	7.6 \pm 2.6	6.0 \pm 1.5	0.9 \pm 0.2
Intestine wall	19.4 \pm 9.6	11.2 \pm 1.7	1.3 \pm 0.4
Kidney	12.3 \pm 2.3	11.1 \pm 1.5	3.4 \pm 0.4
Liver	14.4 \pm 5.9	11.5 \pm 2.2	2.8 \pm 1.2
Skeleton	12.8 \pm 8.5	11.8 \pm 3.4	2.4 \pm 1.8
Spinal cord	8.4 \pm 4.3	11.2 \pm 6.1	0.8 \pm 0.2
Spleen	7.9 \pm 4.0	9.2 \pm 1.5	4.7 \pm 3.4
Stomach wall	7.7 \pm 4.2	6.1 \pm 1.3	1.0 \pm 0.3
Swim bladder wall	9.2 \pm 10.2	5.8 \pm 2.0	1.2 \pm 0.5
White fat	67.6 \pm 35.9	61.0 \pm 5.3	15.2 \pm 10.0

SD: standard deviation (n= 3 fish).

The highest concentration of TRR was determined in white fat with a mean concentration of 67.6 mg eq/kg at 35 days post-dose. Lufenuron is excreted as unchanged compound in the faeces. The TRR data indicate a slow depletion of radioactivity.

Quantitative whole body autoradiography (QWBA) analysis of longitudinal fish sections indicated that lufenuron is located primarily in the fat and viscera. No metabolism of lufenuron in Atlantic salmon was detected over the six-month period.

Fillet and pooled faecal samples were extracted with acetonitrile (2 x 10 mL) for metabolite profiling using HPLC with LSC detection. Unextracted residues (TRR) were in fillet: 1.1 % (day 1 post-dose), 1.4 % (day 30 post-dose), 8.8 % (day 90 post-dose) and 3.4 % (day 178 post-dose), and in faeces 3.3 %.

No metabolites were detected over the 6-month period after medication. In the chromatograms, only one peak was observed and identified as the parent compound. This result was also confirmed by TLC. No polar metabolites were detected.

In vivo metabolism in fish

Bluegill

In a GLP-compliant study (Forbis, 1987) one hundred and twenty bluegill (*Lepomis macrochirus*), with an initial mean weight of 5.4 g (± 1.5 g), were exposed to [^{14}C]-lufenuron (specific activity 31.7 $\mu\text{Ci}/\text{mg}$, purity higher than 98 %, labelled at the difluorophenyl ring) in water at a concentration of 13 $\mu\text{g}/\text{L}$ over 49 days at 22 °C (equivalent to 1078 degree-days). The water (100 L in the aquaria) was replenished in a continuous flow system and the test substance was added to the water with the aid of a small quantity of acetone (0.1 mL). After the 49-day period fish were transferred to clean water for 28 days to evaluate the depuration. Fillet, viscera and whole fish were sampled at days 0, 0.17, 1, 3, 7, 14, 21, 28, 35, 42 and 49 for the uptake phase and at days 1, 3, 7, 10, 14, 21 and 28 for the depuration phase. Water was sampled at the same days for analysis. Radioactivity was measured by combustion and liquid scintillation counting. Daily bioconcentration factors ranged from 11 to 2500X, 29 to 4000X and 45 to 6800X for fillet, whole fish and viscera, respectively. Uptake concentration ranged from 0.19 to 25 mg eq/kg for fillet, 0.52 to 40 mg eq/kg for whole fish and 0.81 to 68 mg eq/kg for viscera. Radioanalysis throughout the depuration phase indicated a decrease of 50 %, 42 % and 46 % of lufenuron from fillet, whole fish and viscera, respectively.

For metabolic profiling, fish tissues were sampled from day 49 of the uptake period (Leak, 1987). Characterization was performed by TLC in two different solvent systems. The TRR was completely extracted with dichloromethane and only lufenuron was detected.

Fathead minnow

In a similar GLP-compliant study (Maynard *et.al.*, 2004), four hundred and sixteen fathead minnow (*Phimephales promelas*), with a weight in the range of 2.1 to 6.3 g, were exposed to [^{14}C]-lufenuron (specific activity 1.8 MBq/ mg, purity of 95.7 %, labelled at the dichlorophenyl ring) in water at nominal [^{14}C]-lufenuron concentrations of 1 and 10 $\mu\text{g}/\text{L}$ over 60 days at 25 °C

(equivalent to 1500 degree-days). After this period, fish were transferred to clean water for the depuration phase, that comprised 60 days at 25 °C or 1500 degree-days. The water (120 L in the aquaria) was replenished in a continuous flow system and the test substance was dissolved in triethylene glycol. Viscera, flesh, carcass and whole fish were sampled at days 7, 14, 21, 28, 35, 42, 49, 52, 54, 58 and 60 for the uptake phase and at days 1, 3, 8, 15, 22, 29, 36, 43, 57 and 60 for the depuration phase. Total radioactive residues in the viscera, flesh, carcass and whole fish were analysed during the uptake and depuration phases. The uptake rate constants were calculated to be 936 day⁻¹ and 644 day⁻¹ for the 1 and 10 µg/L of [¹⁴C]-lufenuron treatments, respectively. During the depuration phase the concentration of [¹⁴C]-lufenuron in the whole fish decreased steadily. Within 29 days the concentration dropped from the mean plateau phase concentrations by 49 % and 25 % in the nominal 1 and 10 µg/L treatments, respectively. After 60 days, 83 % and 79 % depuration had been achieved for the lower and higher [¹⁴C]-lufenuron concentrations. The depuration constants were calculated to be 0.0335 day⁻¹ and 0.0239 day⁻¹ for the 1 and 10 µg/L of [¹⁴C]-lufenuron treatments, respectively. The kinetic bioconcentration factor, calculated as the ratio of the uptake rate constant to the depuration rate constant, were 28000 and 27000, for the lower and higher [¹⁴C]-lufenuron concentrations, respectively.

Fish analysis by TLC confirmed that the residues in fish tissues were predominantly lufenuron (91 %-96 %) and, therefore, no metabolite characterisation was performed. The mean recovery of radioactivity in the acetonitrile extracts analysed by TLC was in the range of 82 to 106 %.

Comparative metabolism

The metabolic pathway of lufenuron is similar in rodents (rats), ruminants (goat) and poultry (chicken). Lufenuron is metabolized to a very limited extent by cleavage of the benzoyl ureido bridge leading to 1-(2,5-dichloro-4-(1,1,2,3,3,3-hexafluoropropoxy)phenyl)urea and 2,6-difluorobenzoic acid and by cleavage of the urea bridge leading to the 2,6-difluorobenzamide. The same minor metabolites were identified in tissues, eggs, milk and excreta. Some minor uncharacterized metabolites were detected in goat excreta and liver and in hen excreta, kidney and egg white. Important routes of elimination were milk in lactating goats and egg yolk in laying hens.

Lufenuron is not metabolized in bluegill sunfish and fathead minnow, independent of the label of the molecule; the only residue was the parent compound. In Atlantic salmon, also no metabolites were detected and lufenuron is only slowly eliminated. The main route of elimination in fish is via faeces and lufenuron is mainly distributed in fat tissues.

Tissue residue depletion studies

Radiolabelled residue depletion studies

Salmon

In a GLP-compliant study (Hobbs, 2014), conducted at a water temperature of 4 to 14 °C, [¹⁴C]-lufenuron (specific activity 95.5 µCi/mg, purity higher than 99.8 %, labelled at the acyl carbon adjacent to the difluorophenyl ring) at an intended dose equivalent to 5 mg/kg bw (actual dose 5.34 mg/kg bw) was administered in feed to 64 post-smolt salmon (*Salmo salar* L.) (107-185 g) held in tanks supplied with seawater and aerated via air stones.

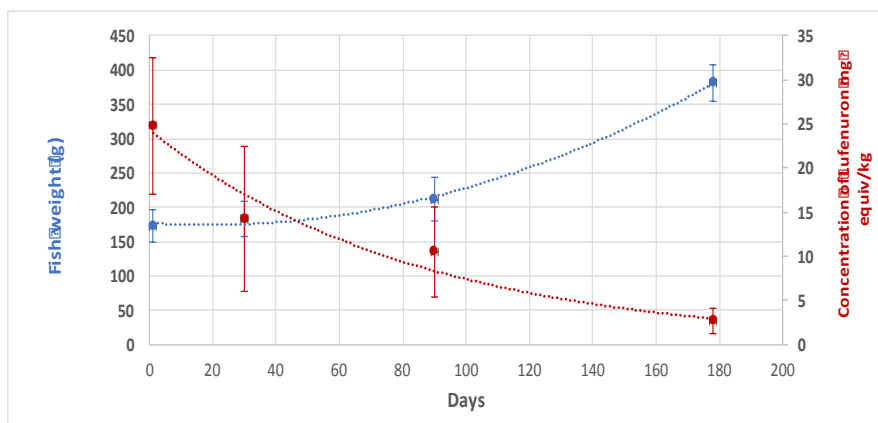
Ten fish were sacrificed at 1 day, 30 days, 90 days and 178 days after the end of treatment and muscle plus skin (fillet) sampled for analysis. TRR was determined by LSC using Premafluor[®]E scintillation fluid. Acetonitrile extracts of salmon fillets were analysed by HPLC with a radiochemical detector. Combustion efficiencies were higher than 97 %. The limit of detection for TRR and chromatography were assessed to be < 0.01 mg/kg. TRR was almost completely extracted from fillet and the parent compound was the only residue detected in salmon fillet (Table 10).

The highest concentration of radioactivity in fillet was determined 1 day after end of the treatment phase.

Lufenuron was the only residue detected in fish faeces. Extraction of the combined faecal samples released 96.8 % of the TRR (5.36 mg eq/kg). Radioactive residues in the faeces declined from about 7 mg eq/kg to 2 mg eq/kg at the end of the in-life phase of the study.

Smolting and migration of Atlantic salmon from freshwater to saltwater is energy demanding and affects the growth rate. Atlantic salmon have a period of low growth rate followed by a period of excessive growth (compensatory growth). In general, smolts transferred to seawater cages reach harvest size of 4 kg in 10 – 15 months. However, in this study the growth of fish was impaired, they only doubled their body weight in 6 months, which is characteristic of fish held in confined tanks. Therefore, the data of this study are not adequate to be considered for a depletion study. However, the data indicate that the concentration of lufenuron decreases with time in the same manner as the fish body weight increases (Figure 8). These results suggest that lufenuron is distributed in the fatty fish tissues rather than eliminated.

Figure 8. Fish body weight and lufenuron depletion in Atlantic salmon fillet following a total dose of 5.34 mg/kg bw



Residue depletion studies with unlabelled drug

Salmon

Six GLP-compliant studies were conducted with unlabelled lufenuron in different geographic locations: Canada (three studies), Chile (one study) and Norway (two studies) (Table 10).

Atlantic salmon smolts (mixed sex), held in a freshwater hatchery, were treated with lufenuron via feed at a nominal dose of 5 mg/kg bw for seven consecutive days. Fish were transferred to sea cages after the end of medication and remained at sea until the end of the study. Water temperatures varied according to the geographic site where the studies were conducted. Following treatment, fish were transferred to sea cages and kept at sea until the end of the studies. Fish were harvested at various time points after treatment.

Table 10. Summary of the residue depletion studies carried out in different geographic locations

Country of the study	Study number/ Study report	Season (Sea transfer)	Temperature (°C)		Reference
			Hatchery	Sea	
Canada	GSO-13-005/ ATL-14-1123	Autumn	10.3	1.7-13.7	Hervy, 2015b
Canada	NAH-13-071/ ATL-15-1351	Autumn	10	0.7-13.2	Hervy, 2016b
Canada	NAH-15-014/ ATL-15-1430	Spring	13	4-16	Hervy, 2016c
Chile	CHI-12-002	Spring	11	9-18	Lewis, 2015
Norway Voldness sea	NAH-14-114/ ATL-15-1352	Autumn	10-12	5.8-15.6	Hervy, 2015d
Norway Langholmen sea	NAH-14-114/ ATL-15-1363	Spring	3.5	3.7-13.3	Hervy, 2016a

Residue depletion data were obtained using the entire fillet from one side of the fish including skin, scales and belly flap. Lufenuron was quantified in fillet using a validated UHPLC-MS/MS method. Fluazuron (N-((4-chloro-3-((3-chloro-5-(trifluoromethyl)pyridin-2-yl)oxy)phenyl)carbamoyl)-2,6-difluorobenzamide) was used as internal standard. The sample preparation procedure consisted of extraction of lufenuron from minced and homogenized fillet by solid-liquid extraction with acetonitrile. The clean-up of the extracts was performed by solid phase extraction on C18 sorbents. Recoveries were above 90 % and the limit of quantitation of the method was 50 µg/kg.

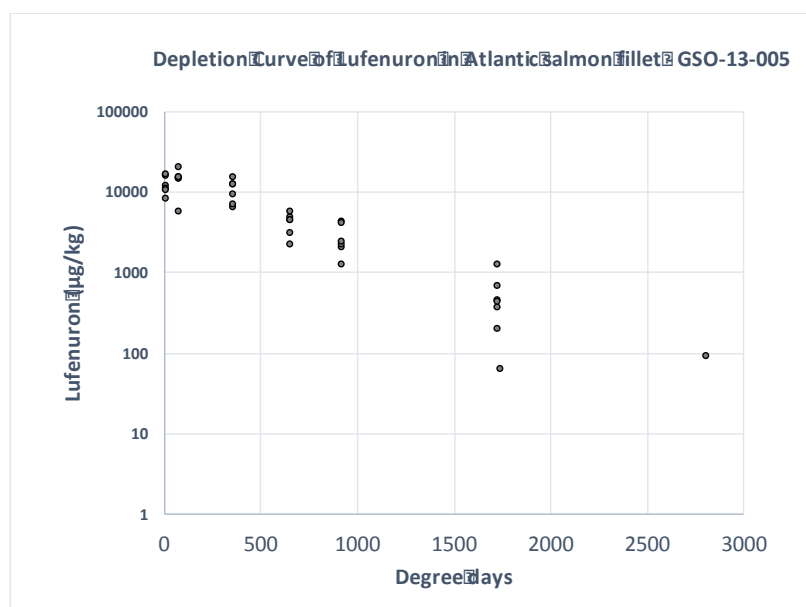
Study GSO-13-005

In a GLP-compliant study carried out in Canada (Hervy, 2015b), Atlantic salmon smolts (average weight of 115 g, nine days prior the start of treatment) were fed lufenuron in the diet for seven days at an intended dose of 5 mg/kg bw (actual total dose of 33.9 mg/kg bw). The water temperature at sea varied in the range of 1.7 °C to 13.7 °C. Six fish were sampled at each time point (1, 8, 37, 96, 192, 283 and 369 days post-dose) and fillet was collected, with exception of the last time point (369 days) where only two fish were sampled. Lufenuron was quantified by a validated UHPLC-MS/MS method. The mean concentrations of lufenuron in fillet are shown in Table 11.

Table 11. Mean concentration of lufenuron in fillet from Atlantic salmon with time after oral daily dose (medicated feed) of lufenuron (5 mg/kg bw) for 7 days (Hervy, 2015b)

Time post-dose (d)	Time post-dose (DD)	T (°C)	Number of fish	Concentration of lufenuron (µg/kg)			Standard deviation (µg/kg)
				Mean	Minimum	Maximum	
1	20.7	10.3	6	12315	8090	16600	3342
8	85.3	5.7	6	14223	5640	20400	4766
37	364.4	8.4	6	10428	6300	15400	3594
96	658.6	3.1	6	4088	2170	5590	1261
192	925.7	4.8	6	2693	1270	4240	1187
283	1730	13.2	6	564	201	1270	378
369	2814	12.3	2	77	64	91	19

Figure 9 shows the concentration of lufenuron in Atlantic salmon versus days post-dose from the depletion study carried out in Canada.

Figure 9. Concentration of lufenuron in Atlantic salmon fillet versus days post-dose

The initial half-life of elimination calculated from the residue data from days 1 to 369 in the fillet was 359 degree-days (EMEA 1999).

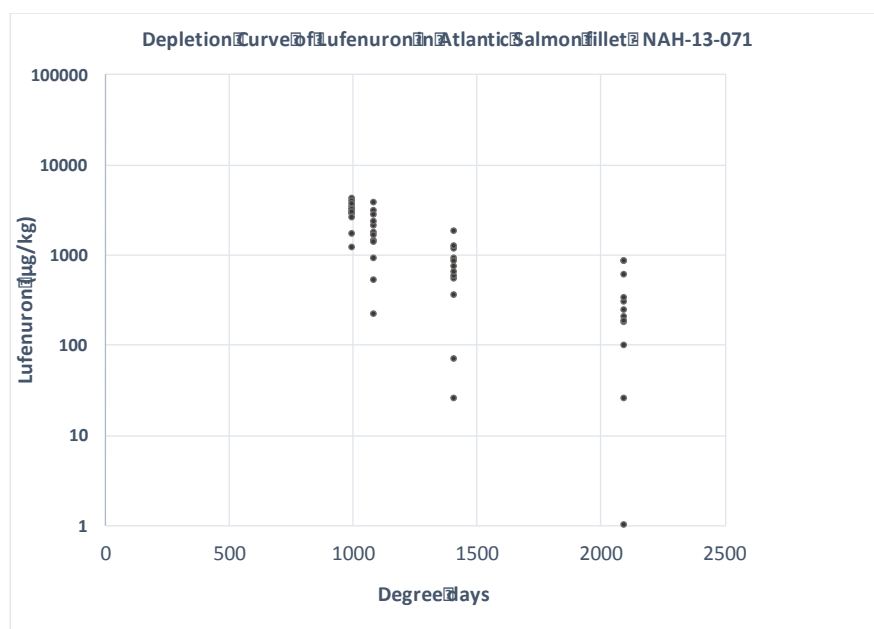
Study NAH-13-071

In another GLP-compliant study conducted in Canada (Hervy, 2016b), Atlantic salmon smolts (average weight at the start of treatment, 88 g) were fed lufenuron in the diet for seven days at an intended dose of 5 mg/kg bw (actual total dose of 34.2 mg/kg bw). The water temperature at sea varied in the range of 0.7 °C to 13.2 °C. Tissues were collected after 127, 187, 247 and 307 days post-treatment. Twelve fish were sampled for each time point. Lufenuron was quantified by a validated UHPLC-MS/MS method. The mean concentrations of lufenuron in fillet are shown in Table 12 and the depletion curve in Figure 10.

Table 12. Mean concentration of lufenuron in fillet from Atlantic salmon with time after oral daily dose (medicated feed) of lufenuron (5 mg/kg bw) for 7 days (Hervy, 2016b)

Time post-dose (d)	Time post-dose (DD)	Number of fish	Concentration of lufenuron (µg/kg)			Standard deviation (µg/kg)
			Mean	Minimum	Maximum	
127	999	12	3023	1190	4200	858
183	1088	12	1964	217	3680	958
246	1407	12	776	25	1820	464
308	2099	12	324	25	851	283

Two samples presented a lufenuron concentration lower than the LOQ. These values were reported as half of LOQ (25 µg/kg).

Figure 10. Depletion curve of lufenuron in salmon filletStudy NAH-15-014

In another GLP-compliant study conducted in Canada (Hervy, 2016c), Atlantic salmon smolts (average weight three days prior to the start of treatment, 62 g) were fed with lufenuron diet for seven days at an intended dose of 5 mg/kg bw (actual total dose of 33.8 mg/kg bw). The seawater temperature ranged from 4 °C to 16 °C. The fish growth in the hatchery had been delayed due to the unusually low temperature of the fresh water. Due to logistical considerations, the smolts were divided into two batches (Endris *et.al.*, 2017). Fillet samples were collected after 2, 4, 6 and 8 months post-treatment and lufenuron was quantified by a validated UHPLC-MS/MS method. The mean concentrations of lufenuron in fillet are shown in Table 13. Unlike the previous study results, it was not possible to establish the depletion curve for this study due to the difficulty in assigning correct degree-days due to the sampling times being reported in months.

Table 13. Mean concentration of lufenuron in fillet from Atlantic salmon with time after oral daily dose (medicated feed) of lufenuron (5 mg/kg bw) for 7 days (Hervy, 2016c)

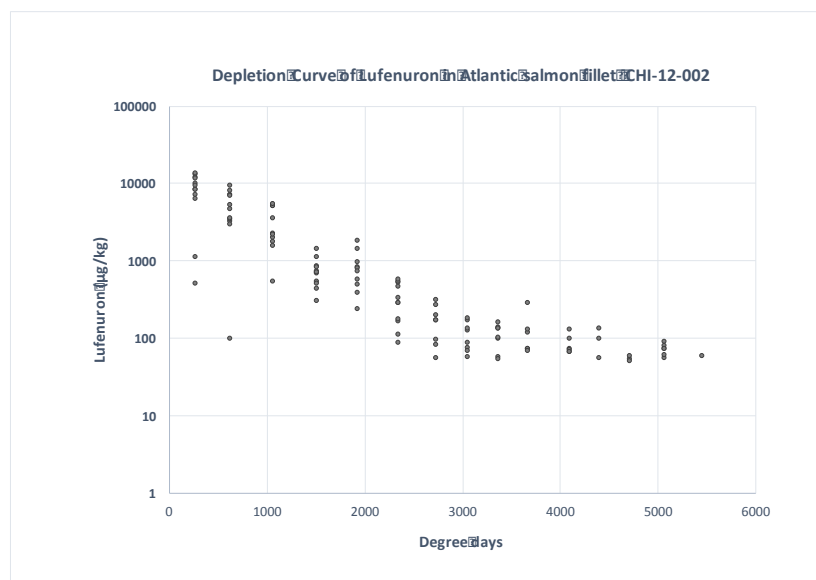
Time post-dose (months)	Number of fish	Concentration of lufenuron (µg/kg)			Standard deviation (µg/kg)
		Mean	Minimum	Maximum	
2	18	3659	<LOQ	8330	2295
4	18	492	<LOQ	965	342
6	18	96	<LOQ	149	78
8	17	110	<LOQ	177	56

Study CHI-12-002

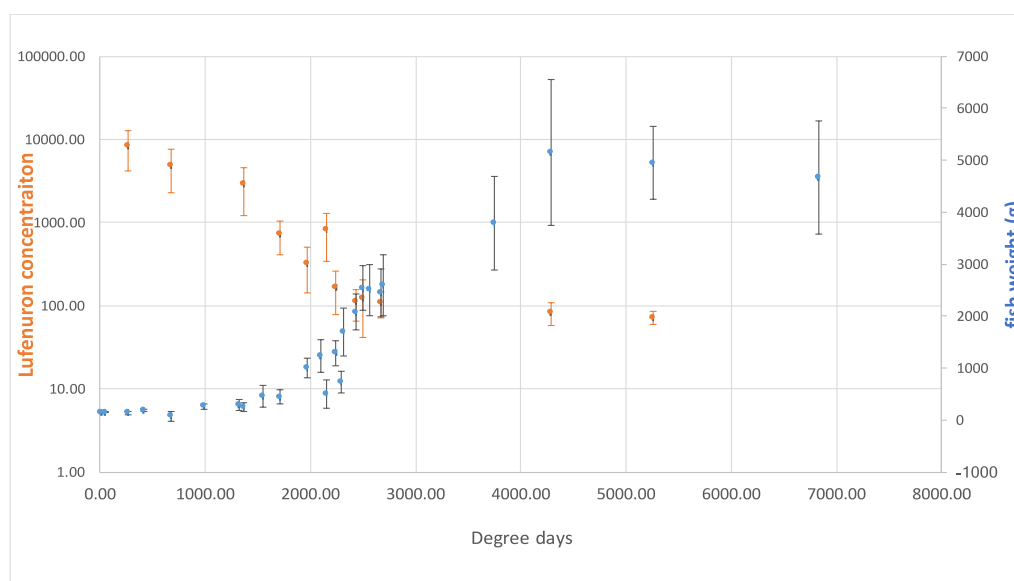
In a GLP-compliant study carried out in Chile (Lewis, 2015), Atlantic salmon smolts (average weight prior the start of treatment, 125 g, mixed sex), held in freshwater hatchery tanks (water temperature of 11 °C), were fed with lufenuron diet for seven days at an intended dose of 5 mg/kg bw (actual total dose of 33.9 mg/kg bw). Six days post end of the treatment fish were transferred to sea cages. The seawater temperature ranged from 4 °C to 16 °C. Tissues were collected after 1, 4, 21, 49, 76, 104, 132, 160, 189, 217, 246, 274, 315, 343, 371, 401 and 430 days post-treatment. Fish were sampled for each time point and lufenuron quantified by a validated UHPLC-MS/MS method. The mean concentrations of lufenuron in fillet are shown in Table 14 and the depletion curve is shown in Figure 11.

Table 14. Mean concentration of lufenuron in fillet from Atlantic salmon with time after oral daily dose (medicated feed) of lufenuron (5 mg/kg bw) for 7 days (Lewis, 2015)

Time post-dose (d)	Time post-dose (DD)	T (°C)	Number of fish	Concentration of lufenuron (µg/kg)			Standard deviation (µg/kg)
				Mean	Minimum	Maximum	
21	265.5	12.6	12	8349	510	13300	4178
49	620.6	12.7	11	4908	98	9210	2651
76	1063	14.0	10	2908	538	5310	1700
104	1516	14.6	11	734	298	1430	320
132	1932	14.6	10	813	235	1790	473
160	2351	14.7	11	322	87	570	179
189	2728	14.4	8	167	55	309	90
217	3052	14.1	8	111	56	177	47
246	3373	13.7	8	109	54	159	38
274	3667	13.4	6	123	68	281	81
315	4099	13.0	6	83	65	128	25
343	4405	12.8	3	94	55	131	38
371	4720	12.7	3	54	50	58	4
401	5070	12.6	6	72	55	90	13
430	5457	12.7	1	59	59	59	

Figure 11. Depletion curve of lufenuron with time in degree-days

It is possible to verify that the concentration of lufenuron decreases as the fish weight increases (Figure 12). The Atlantic salmon were medicated when they still are in freshwater (smolts, with a body weight about 100 g). When they are transferred to sea cages the growth rate is affected and very limited in the first period (until 1000 degree-days). In the second phase, an excessive growth rate is observed at the same time that the concentration of lufenuron decreases. Even some depletion occurs because lufenuron is detected in faeces, the concentration in fillet remains high. As fish increases in size, the percentage of fat in the fillet also increases which may explain the low elimination rate of lufenuron. About 55 % of the fillet is fat and lufenuron is a very lipophilic compound.

Figure 12. Concentration of lufenuron and fish body weight in function of time post-dose of lufenuron to Atlantic salmon

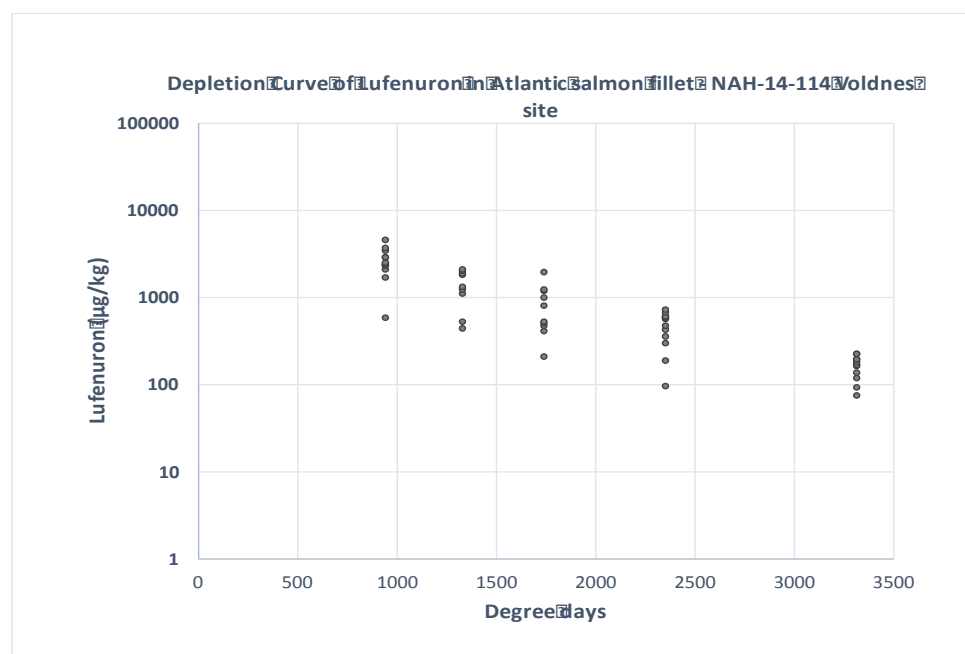
Study NAH-14-114 (Voldnes Sea)

In a GLP-compliant study carried out in a commercial fish farm (Voldnes) in southern Norway (Hervy, 2015d), Atlantic salmon smolts (average weight at the start of treatment, 102 g, mixed sex), held in freshwater hatchery tanks (water temperature of 10-12 °C), were fed with lufenuron diet for seven days at an intended dose of 5 mg/kg bw (actual total dose of 36.2 mg/kg bw). Six days post end of the treatment fish were transferred to sea cages and maintained in seawater until end of the study. The seawater temperature ranged from 5.8 °C to 15.6 °C. Fillet samples were collected after 104, 167, 222, 246, 279 and 347 days post-treatment. Twelve fish were sampled for each time point and lufenuron quantified by a validated UHPLC-MS/MS method. Lufenuron was quantified in fish fillet and the mean concentrations are shown on Table 15.

Table 15. Mean concentration of lufenuron in fillet from Atlantic salmon with time after oral daily dose (medicated feed) of lufenuron (5 mg/kg bw) for 7 days (Hervy, 2015d)

Time post-dose (d)	Time post-dose (DD)	Number of fish	Concentration of lufenuron (µg/kg)			Standard deviation (µg/kg)
			Mean	Minimum	Maximum	
104	944	12	2498	1980	4300	843
167	1335	12	1255	423	1920	526
222	1743	12	862	393	1860	547
279	2358	12	446	284	688	154
347	3318	12	158	72	214	56

The maximum lufenuron concentration was determined in the first-time point (4300 µg/kg). The fish weights were not provided in this study; only the weight and length of the fillets were given for each sample. The data fit well to the log-e transformed regression model and the ln-linearity, normal distribution of errors and homogeneity of variances assumptions were satisfied. The depletion curve is shown in Figure 13.

Figure 13. Depletion curve of lufenuron in salmon fillet

Study NAH-14-114 (Langolmen Sea)

In a GLP-compliant study carried out in a commercial fish farm (Langholmen) in northern Norway (Hervy, 2016a), Atlantic salmon smolts (average weight at the start of treatment, 98 g, mixed sex), held in freshwater hatchery tanks (water temperature of 3.5 °C), were fed with lufenuron diet for seven days at an intended dose of 5 mg/kg bw (actual total dose of 35.0 mg/kg bw). One to three weeks post end of the treatment fish were transferred to seawater cages and maintained in seawater until the end of the study. The seawater temperature ranged from 3.7 °C to 13.3 °C. Tissues were collected after 62, 127, 184 and 246 days post treatment. Twelve fish were sampled for the first two time points, eleven for the third time point and ten for the last time point. Lufenuron was quantified in the collected fillet samples by a validated UHPLC-MS/MS method. The mean concentrations of lufenuron determined are shown on Table 16.

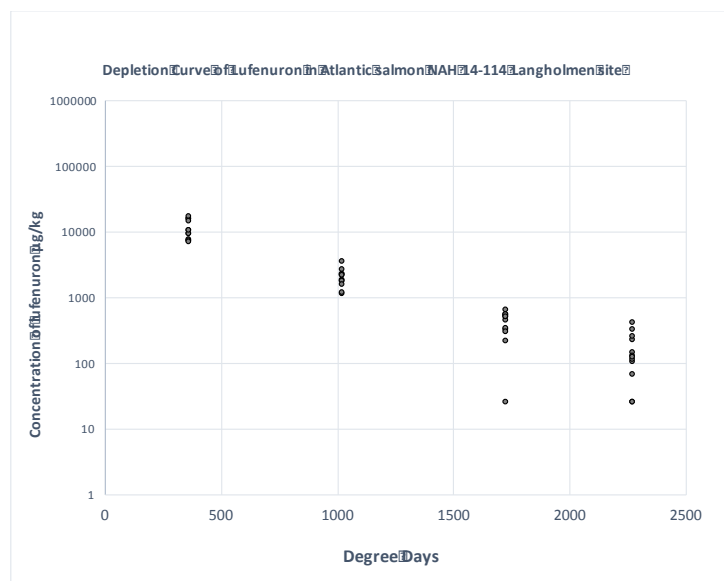
Table 16. Mean concentration of lufenuron in fillet from Atlantic salmon with time after oral daily dose (medicated feed) of lufenuron (5 mg/kg bw) for 7 days (Hervy, 2016a)

Time post-dose (d)	Time post-dose (DD)	Number of fish	Concentration of lufenuron (µg/kg)			Standard deviation (µg/kg)
			Mean	Minimum	Maximum	
62	362	12	10891	7240	15900	3858
127	1024	12	2010	1760	2670	358
184	1725	12	438	321	553	88
246	2273	12	178	25	412	149

The concentration of lufenuron as a function of time in degree-days is shown in Figure 14. Using all data points, it was verified that the residues are not normally distributed and are not

constant over time. After excluding three results (below of LOQ) the data fitted the method of least square.

Figure 14. Depletion curve of lufenuron in salmon fillet



The depletion curves of the studies carried out in Canada, Norway and Chile are summarized in Figure 15 and the log-e transformed curves in Figure 16.

Figure 15. Depletion curves of all studies (except study NAH 15-014)

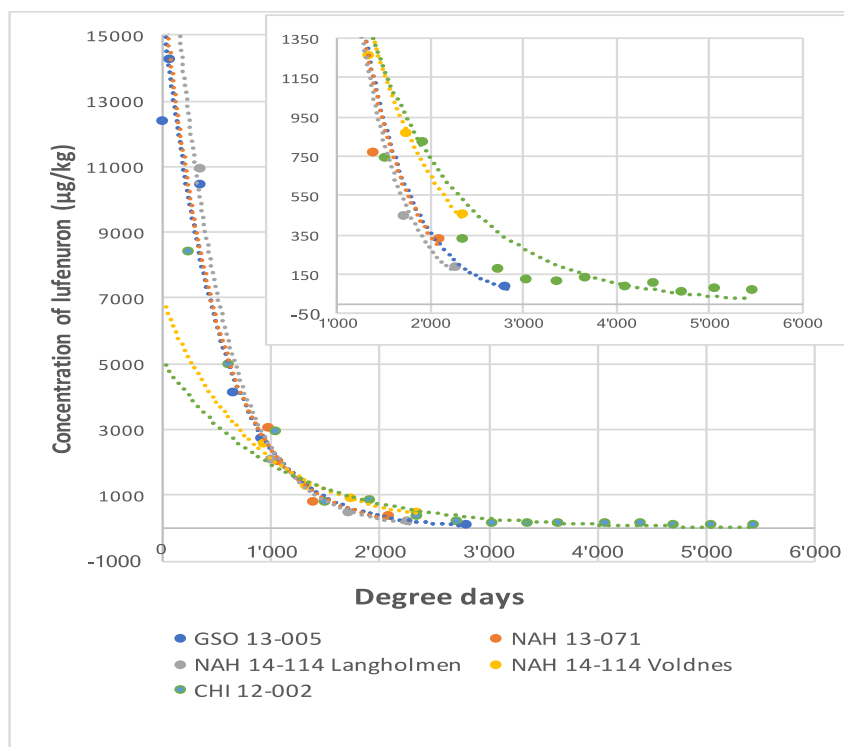
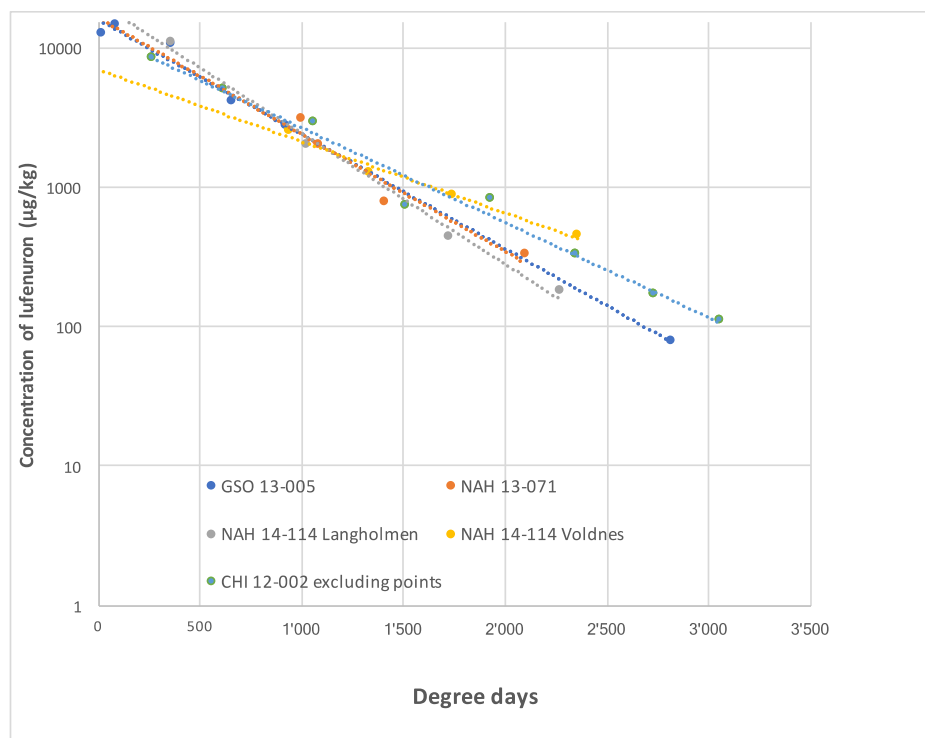


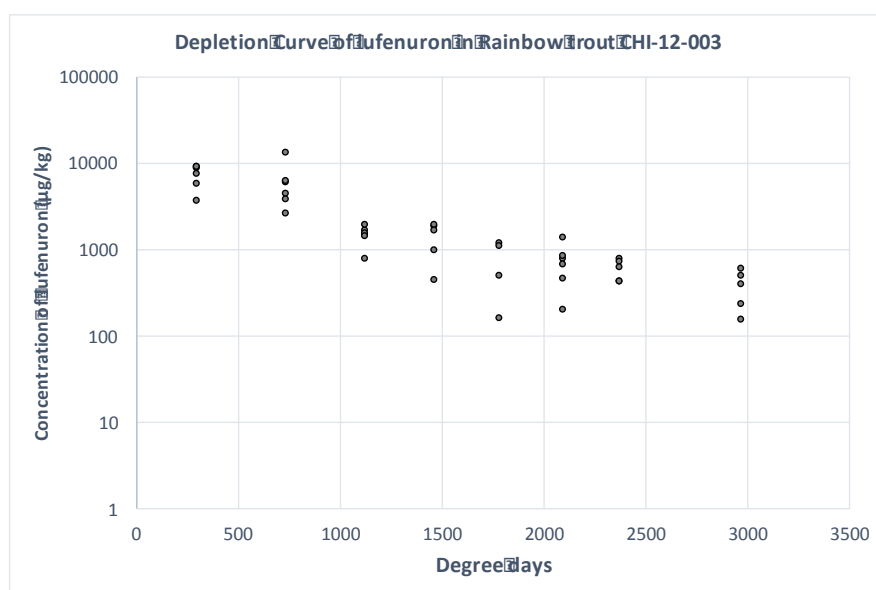
Figure 16. Depletion curves (log-e transformed) of all studies (except study NAH 15-014)

Trout

In a GLP-compliant study carried out in Chile (Lewis *et.al.*, 2017), rainbow trout smolts (*Oncorhynchus mykiss*) (average weight at the start of treatment, 212 g, mixed sex), held in freshwater hatchery tanks (water temperature of 11.2 °C), were fed with lufenuron diet for seven days at a dose of 5 mg/kg bw (actual total dose of 35.0 mg/kg bw). Eight days post end of the treatment fish were transferred to sea cages and maintained in seawater until end of the study. The seawater temperature ranged from 9.2 °C to 17.1 °C. Whole fillet samples were collected after 21, 49, 78, 106, 134, 163, 190 and 244 days post-treatment. Four to six fish were sampled for each time point and lufenuron was quantified in the collected fillet samples by a validated UHPLC-MS/MS method. The mean, maximum and minimum concentrations of lufenuron determined are shown on Table 17 and the depletion curve in Figure 17.

Table 17. Mean concentration of lufenuron in fillet from rainbow trout with time after oral daily dose (medicated feed) of lufenuron (5 mg/kg bw) for 7 days (Hervy, 2016a)

Time post-dose (d)	Time post-dose (DD)	Number of fish	Concentration of lufenuron (µg/kg)			Standard deviation (µg/kg)
			Mean	Minimum	Maximum	
21	300	6	7142	3600	9000	2148
49	736	6	5892	2540	12800	3635
78	1126	6	1451	763	1910	381
106	1467	5	1359	444	1913	633
134	1780	4	728	160	1180	485
163	2094	6	711	196	1370	396
190	2375	5	586	415	766	164
245	2967	5	372	153	592	181

Figure 17. Depletion of lufenuron in Rainbow trout in function of time (degree-days)

Method of analysis for residues in tissues

Due to their low volatility and thermal instability benzoylurea insecticides, including lufenuron, are usually determined in food, feed and biological matrices by liquid chromatography. There are no methods reported in the scientific literature about the determination of lufenuron in fish. Some methods are reported for the determination of residues of lufenuron in agricultural commodities and environmental matrices.

Liquid chromatography

Analytical methods based on the quantitation of lufenuron in plants and food of animal origin by high performance liquid chromatography with an ultraviolet detector were described in the JMPR evaluation (2015) of pesticide residues in food. In general, lufenuron was extracted by

solvents and the extracts cleaned-up by solid phase extraction using silica gel, cyano or C18 sorbents. For samples rich in proteins, such as milk, a protein precipitation step is introduced in the procedure. The quantitation of lufenuron is performed at the wavelength of 255 nm. The limit of quantitation is about 0.01 mg/kg for plant and 0.02 mg/kg for animal commodities.

Confirmatory methods

Liquid chromatography – tandem mass spectrometry (LC-MS/MS)

An ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS) method was validated in compliance with GLP to determine residues of lufenuron in fish fillet (muscle and skin in natural proportions) from salmon and rainbow trout in the studies submitted to the current Meeting of the Committee (Adams, 2013). This original method was revalidated in another laboratory for the determination of lufenuron in salmon fillet (Hervy, 2013c) and trout fillet (Hervy, 2013d). The Committee assessed the validation data against the requirements for analytical methods as published in Codex Guideline CAC/GL 71-2009 (FAO/WHO, 2014).

In summary, the sample preparation consists of addition of fluazuron (N-((4-chloro-3-((3-chloro-5-(trifluoromethyl)pyridin-2-yl)oxy)phenyl)carbamoyl)-2,6-difluorobenz- amide) as internal standard to the mechanically homogenized tissues (0.5 g) followed by solid-liquid extraction with acetonitrile (5 mL). After centrifugation (15 min), the supernatant is diluted with 10 mL water and percolated on a solid phase extraction cartridge containing a C18 sorbent. The cartridge is washed with 2 mL acetonitrile:water 30:70 v/v, dried under vacuum and the retained analytes eluted with 10 mL acetonitrile:water 70:30 v/v. The eluate is filtered and 10 µL injected into the UHPLC-MS/MS. The separation of lufenuron and the internal standard is performed on a Waters Acquity CSH C18 column (2.1 x 50 mm, 1.7 µm), at 40 °C, with a mobile phase containing 0.05 % v/v formic acid (solvent A) and acetonitrile (solvent B) at a flow rate of 0.4 mL/min, under gradient elution (0 to 0.3 min, 80:20 v/v A:B to 55:45 v/v A:B; 1.6 min 50:50 v/v A:B; 3.1 min 0:100 v/v A:B; 6.8 min 0:100 v/v A:B and 7 min 80:20 v/v A:B). The mass spectrometer conditions were: electrospray source operating in the negative mode, capillary voltage of 3.25 kV, source temperature of 120 °C, desolvation temperature of 280 °C, cone gas flow of 100 L/h and desolvation gas flow of 700 L/h. Quantitation is performed using acquisition of ions in the selected reaction-monitoring mode, using the transitions of m/z 508.9→326.0 for lufenuron and m/z 504.1→305.1 for fluazuron. For identification and identity confirmation two additional transitions for lufenuron were monitored: m/z 508.9→174.0 and m/z 508.9→201.9. A plot of the area response ratio of lufenuron to fluazuron versus concentration of lufenuron was fitted to a quadratic regression model with 1/x weighting. Linearity was observed in the range of 2.5 to 200 ng/mL equivalent to 50 to 4000 µg/kg.

The LOD and LOQ were estimated as the signal to noise ratio of 3 and 10, respectively, at the retention time of lufenuron. The matrix effect and extraction recovery were evaluated at the LOQ, using at least six different batches of control matrix. The method validation parameters, using fortified samples, are presented in Table 18.

Table 18. Validation parameters of the UHPLC-MS/MS method for the determination of lufenuron in salmon tissues (Adams, 2013)

Parameter	Salmon fillet	Trout fillet
Interday precision (CV, n=18)	8.6 % (100 µg /kg)	5.7 % (1500 µg /kg)
	9.4 % (600 µg /kg)	
	7.2 % (1500 µg /kg)	
	6.4 % (3000 µg /kg)	
Accuracy (n=18)	102 % (100 µg /kg)	97.7 % (1500 µg /kg)
	101 % (600 µg /kg)	
	97.6 % (1500 µg /kg)	
	102 % (3000 µg /kg)	
LOQ	18 µg/kg (100 µg/kg) ^a	21 µg/kg (100 µg/kg) ^a
LOD	5 µg/kg	6 µg/kg
Analytical range (µg/kg)	50 - 4000	50 - 4000
Linearity (r)	>0.99	>0.99
Specificity/selectivity	No interference observed	No interference observed
Extraction recovery	93.4 %	94.8 %
Matrix effect	+19.4 %	+5.6 %

a: For practical reasons, regarding a proposed MRL of 1350 µg/kg, the LOQ of the method was set as 100 µg/kg for both fish species.

For the selectivity test, possible interference of other veterinary drugs was assessed: emamectin benzoate, oxytetracycline, teflubenzuron, amoxicillin, deltamethrin, diflubenzuron, florfenicol, azamethiphos and cypermethrin. No interference was observed on the determination of lufenuron in the presence of these drugs.

For matrix co-extractives, 20 and 18 control blank samples of salmon and trout were analysed, respectively. No endogenous interferents with concentrations higher than the LOD were verified.

Minor modifications were introduced in the original analytical method described by Adams (2013) regarding to the sample homogenization (using a Turrax[®] instead of a food processor) and sample weight (2.5 g instead of 0.5 g) and the method was revalidated (Hervy, 2015a) for the determination of lufenuron in salmon fillet (Hervy, 2013c) and trout fillet (Hervy, 2013d). Also, incurred samples were included. The method validation parameters, using fortified samples, are presented in Table 19.

Table 19. Validation parameters of the UHPLC-MS/MS method for the determination of lufenuron in salmon and trout tissues (Hervy, 2015a)

Parameter	Salmon fillet	Trout fillet
Precision, within run (CV)	14.1 % (50 µg /kg)	4.0 % (50 µg /kg)
	2.6 % (600 µg /kg)	3.0 % (600 µg /kg)
	4.0 % (3500 µg /kg)	3.1 % (3500 µg /kg)
Precision, between run (CV)	0 % (50 µg /kg)	8.9 % (50 µg /kg)
	6.6 % (600 µg /kg)	2.5 % (600 µg /kg)
	0 % (3500 µg /kg)	3.2 % (3500 µg /kg)
Accuracy	105.4 % (50 µg /kg)	101.0 % (50 µg /kg)
	102.7 % (600 µg /kg)	106.7 % (600 µg /kg)
	98.9 % (3500 µg /kg)	101.4 % (3500 µg /kg)
Limit of quantitation	50 µg/kg	50 µg/kg
Limit of detection	5 µg/kg	6 µg/kg
Analytical range (µg/kg)	50 - 4000	50 - 4000
Linearity (r)	>0.99	>0.99
Specificity/selectivity	No interference observed	No interference observed

The estimated limit of quantitation was 18 µg/kg and 21 µg/kg for salmon and trout fillet, respectively. However, for this method, the lowest calibration point was 50 µg/kg and this was set as the LOQ.

Fifteen incurred salmon fillet samples, with lufenuron residues ranging from about 600 to 2400 µg/kg, were chosen from two depletion studies and analyzed in triplicate in the same analytical run. Sixty percent of the samples analyzed have a coefficient of variation <10 %. The highest value, from one sample, was 22 %.

The FDA monitors lufenuron in salmon tissues using the same multi-residue pesticide monitoring procedure as for teflubenzuron, described in the FDA Laboratory Information Bulletin 4463 (U.S.FDA, 2016).

The method is fully validated and suitable for the depletion studies summarized above. However, the Committee noted that fluazuron, an approved veterinary drug, could affect the analytical result, and therefore may not be an appropriate internal standard for the method used for monitoring purposes.

Stability of residues

The stock solutions of lufenuron (100 µg/mL) and internal standard (100 µg/mL) prepared in acetonitrile are stable for 12 months at 5 °C. At the same conditions, working standard solutions of lufenuron prepared in acetonitrile:water 30:70 v/v are stable for 4 months. The final extracts diluted in mobile phase was proved to be stable for 4 days at ambient temperature.

Lufenuron is stable in salmon fillet and rainbow trout stored at -20 °C at least for 10 months.

Fortified blank salmon fillets with lufenuron (200 µg/kg) were stable over at least three cycles of freeze (20 h)/thaw (4 h).

Appraisal

Lufenuron has not been previously reviewed by the Committee, but was evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR), in 2015, when an ADI of 0-0.02 mg/kg bw per day was established based on the basis of a NOAEL of 1.93 mg/kg bw 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.

Lufenuron is a benzoyl phenylurea insecticide registered for aquaculture use in the treatment of Atlantic salmon at a dose of 5 mg per kg fish for seven days (full therapeutic dose of 35 mg/kg bw), administered through feed (pelleted diet at a level of 5 g/kg), for control of infestation of sea lice. It is also used for the control of flea infestation in companion animals (cats and dogs) and in agriculture to control a wide range of insect pests.

Metabolism data are available for a variety of animal species, including rats, dogs, goats and chicken. In mammals, lufenuron is only metabolized to a limited extent by cleavage of the benzoyl ureido bridge and the biliary excretion is the main path for elimination.

Metabolic profiling in salmon was available. In fish lufenuron is not metabolized and the parent drug is distributed in the fatty fish tissues; no metabolites were detected over a 6-month period after medication.

Radiolabelled data are available for the depletion of lufenuron in salmon at a water temperature of 4 to 14 °C, following repeated dose. Lufenuron was identified as the marker residue in salmon fillet. Lufenuron is almost quantitatively extracted from fish fillet, indicating there are no bound residues. Based on the results of this study, the Committee identified lufenuron as the marker residue in salmon fillet and determined that a value of 1.0 was appropriate for the MR:TRR.

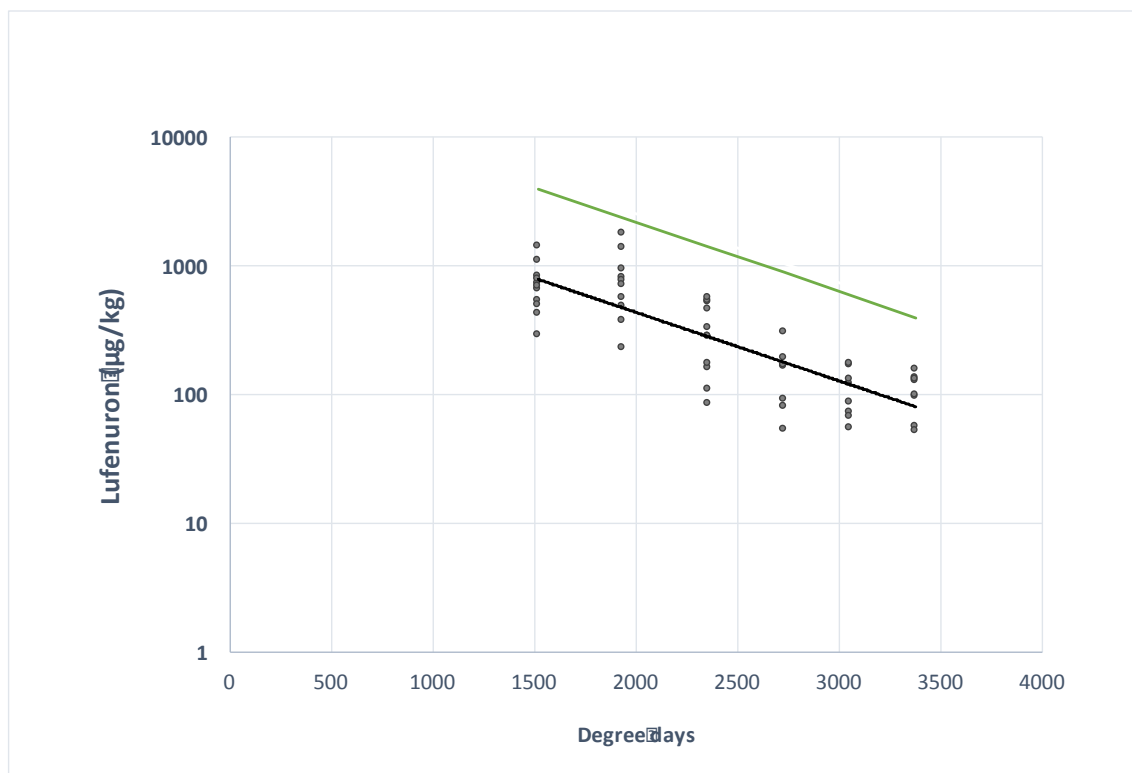
The highest concentration (about 20400 µg/kg) of lufenuron in salmon fillet was registered in one study carried out in Canada 8 days after administration of the drug.

The residue depletion studies in salmon and trout fillets were conducted with a validated method using ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS). The limit of quantification (LOQ) was 50 µg/kg in salmon and trout fillet.

The recommended MRL of 1350 µg/kg of lufenuron in salmon fillet muscle with skin in natural proportions was calculated on the basis of the upper limit of the one-sided 95 % confidence interval over the 95th percentile of total residue concentrations (95/95 UTL) in salmon fillet derived from the data provided, in accordance with Good Veterinary Practice and a withdrawal period of 2050 degree-days.

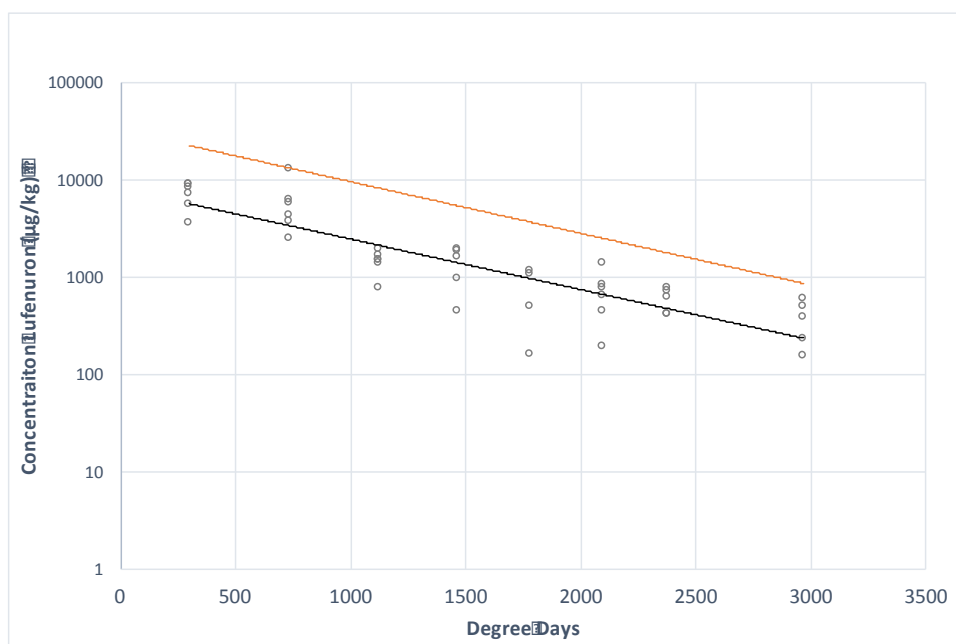
The tolerance limits for lufenuron residues in salmon fillet are shown in Figure 18. The first three and six last time points were excluded.

Figure 18. Tolerance limit considerations for lufenuron in salmon fillet. Regression line (black) and UTL 0.95/0.95 regression line (green)



For rainbow trout, the tolerance limits are shown in Figure 19. The upper limit of the one-sided 95 % confidence interval over the 95th percentile (UTL 95/95) lead to the MRL of 1350 µg/kg at 2643 DD.

Figure 19. Tolerance limit considerations for lufenuron in trout fillet. Regression line (black) and UTL 0.95/0.95 regression line (orange)



Dietary Exposure Assessment

Dietary exposure from pesticide residues (IEDI)

Exposure to lufenuron residues may occur through its use as a pesticide as well as a veterinary drug.

When used as a pesticide, the exposure of lufenuron was 0–4 % of the upper bound of the ADI.

Dietary exposure from veterinary drug residues (GECDE)

When used as a veterinary drug, chronic dietary exposure in the general population was estimated. Based on the toxicological profile of the compound, dietary exposure estimates for children, shorter than lifetime dietary exposure or acute dietary exposure were not required.

Dietary exposure was estimated based on the potential occurrence of residues in salmon and trout muscle and skin in natural proportion. Other finfish were not included in the exposure estimate.

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 of 0.02 mg/kg bw per day (Table 20). Salmon was the major contributor to chronic dietary exposure. It should be noted that no reliable high percentile consumption value was available for trout.

In addition to the accepted GECDE methodology, further calculations were carried out. Instead of using the highest mean and the highest 97.5th percentile consumption across surveys, the calculations were carried out using the mean and the highest reliable percentile for each individual national survey from available data sets (CIFOCCOs). The mean and ranges across surveys were reported (Table 21). The mean of 25 estimates for adults or the general population was 0.35 µg/kg bw per day (2 % of the upper bound of the ADI), with a range of 0.0001–1.1 (<0.01–5.5 % of the upper bound of the ADI).

Combined chronic dietary exposure from pesticide and veterinary drug residues (Extended GECDE)

Modified methods based on the GECDE were used to estimate combined chronic dietary exposure (Table 22). The usual GECDE approach was extended to include additional commodities that were assessed for the compound by JMPR (“Extended GECDE”). It should be noted that this new exposure assessment methodology is still being piloted. It should further be noted that the median residues used as inputs were extracted from JMPR publications and have not been validated for this assessment.

Combined chronic dietary exposure from veterinary drug and pesticide residues was considered for the general population based on the potential occurrence of residues in salmon, trout, mammalian meat, mammalian fats, edible mammalian offal, milks, poultry meat, poultry fats, edible poultry offal, eggs and plant products (cucumber, melons, except watermelon, sweet pepper, potato, tomato, tomato juice and tomato products).

The Extended GECDE for the general population is 1.8 µg/kg bw per day, which represents 9 % of the upper bound of the ADI of 0.02 mg/kg bw per day. Milks (cow and sheep milk) are the major contributors to combined lufenuron chronic dietary exposure.

Table 20. Estimated chronic dietary exposure to lufenuron (GECDE) occurring in salmon and trout muscle and skin in natural proportion

Category	Type	Median concentr ation ¹ (µg/kg)	Mean consumption ² (whole population, g/kg bw/d)	Highest reliable percentile consumption ³ (consumers only, g/kg bw) /[percentile used]	MR:TR ratio	Exposure (µg/kg bw/day)		GECDE ⁴ µg/kg bw/day	ADI%
						mean	97.5th		
General Population									
Fish and seafood	Salmon ⁵	425	0.18	2.6 [97.5]	1	0.077	1.1		
Fish and seafood	Trout ⁵	348	0.0003	n/a	1	0.0001			
TOTAL						0.0001	1.1	1.1	5.5

¹Median concentration at the end of treatment;²Highest mean consumption figures based on whole population considered from the available dataset;³Highest food consumption figures based on consumers only considered from the available dataset;⁴GECDE is the sum of the highest exposure at the highest reliable percentile of consumption for a food and the mean dietary exposures of the other foods;⁵Muscle and skin in natural proportions.

Table 21. National estimates of chronic dietary exposure to lufenuron median residues in adults or the general population, based on GECDE methodology, occurring in salmon and trout muscle and skin in natural proportion

Country	Population group	Study	Estimate of chronic dietary exposure (µg/kg bw per day)	% ADI	Main contributing food
Belgium	Adults	Diet_National_2004	0.64	3.2	Pacific salmon
China	General population	2002 China Nutrition and Health Survey	<0.01	<0.05	Pacific salmon
Czech Republic	Adults	SISP04	<0.01	<0.05	Pacific salmon
Denmark	Adults	Danish_Dietary_Survey	0.20	1.0	Pacific salmon
Finland	Adults	FINDIET_2007	1.09	5.4	Pacific salmon
France	Adults	INCA2	0.29	1.5	Pacific salmon
Germany	Adults	National_Nutrition_Survey_II	0.82	4.1	Pacific salmon
Hungary	Adults	National_Repr_Surv	<0.01	<0.05	Pacific salmon
Ireland	Adults	NSIFCS	0.48	2.4	Pacific salmon
Italy	Adults	INRAN_SCAI_2005_06	0.50	2.5	Pacific salmon
Latvia	Adults	EFSA_TEST	0.33	1.7	Pacific salmon
Netherlands	Adults	DNFCS_2003	0.28	1.4	Pacific salmon
Spain	Adults	AESAN	0.02	0.09	Pacific salmon
Spain	Adults	AESAN_FIAB	0.43	2.1	Pacific salmon
Sweden	Adults	Riksmaten_1997_98	0.11	0.6	Pacific salmon
United Kingdom	Adults	NDNS	0.45	2.3	Pacific salmon
Mean			0.35	1.8	
Minimum			<0.01	<0.05	
Maximum			0.109	5.4	

Table 22. Estimated chronic dietary exposure to lufenuron (GECDE) occurring in salmon and trout muscle and skin in natural proportion

Category	Type	Median residue ¹ µg/kg	Mean consumption ² g/kg bw/d	Percentile consumption ³ g/kg bw/d (percentile used)	Exposure µg/kg bw/day		GECDE ⁴ µg/kg bw/d
					Mean	HRP	
Meat from mammals	Beef and other bovines meat	12	0.96	4.4 (97.5)	0.012	0.053	0.012
other than marine mammals	Goat and other caprines	12	0.006	4.8 (97.5)	0.0001	0.058	0.0001
	Horse and other equines	12	0.019	4.7 (95)	0.0002	0.056	0.0002
	Pork and other porcines	12	0.99	6.3 (97.5)	0.012	0.076	0.012
	Rabbit meat	12	0.067	3.5 (97.5)	0.001	0.042	0.001
	Sheep and other ovines	12	0.16	4.3 (97.5)	0.002	0.052	0.002
Mammalian fats	Cattle fat	300	0.13	0.61 (97.5)	0.038	0.18	0.038
	Goat fat	300	0.00002	0.047 (90)	0.00001	0.014	0.00001
	Pig fat	300	0.082	1.5 (95)	0.025	0.46	0.025
	Sheep fat	300	0.002	0.11 (97.5)	0.0005	0.032	0.0005
Edible offal (Mammalian)	Cattle, liver	25	0.031	3.3 (97.5)	0.001	0.083	0.001
	Pig liver	25	0.041	2.3 (97.5)	0.001	0.057	0.001
	Sheep liver	25	0.009	2.1 (95)	0.0002	0.053	0.0002
	Cattle, kidney	25	0.007	No HRP	0.0002	-	0.0002
	Pig kidney	25	0.004	1.8 (97.5)	0.0001	0.046	0.0001
	Sheep kidney	25	0.002	0.11 (90)	0.0001	0.003	0.0001
	Cattle, offal of, nes	25	0.037	4.0 (97.5)	0.001	0.099	0.001
	Goat, offal of	25	0.001	2.8 (90)	0.00003	0.070	0.00003
	Horse, offal of	25	0.000	No HRP	0.00001	-	0.00001
	Pig, offal of, nes	25	0.061	2.8 (97.5)	0.002	0.071	0.002
	Sheep, offal of, nes	25	0.003	3.0 (97.5)	0.000	0.075	0.0001
Milks	Cow milk	66	4.5	16.9 (97.5)	0.29	1.1	0.29
	Goat milk	66	0.017	No HRP	0.001	-	0.001
	Sheep milk	66	0.012	18.0 (95)	0.001	1.2	1.2
Poultry meat	Chicken meat	0.6	0.62	6.7 (97.5)	0.0004	0.004	0.0004
	Duck meat	0.6	0.055	4.3 (97.5)	0.00003	0.003	0.00003
	Goose meat	0.6	0.012	4.0 (95)	0.00001	0.002	0.00001
	Poultry meat, nes	0.6	0.037	2.4 (97.5)	0.00002	0.001	0.00002
	Chicken fat	27	0.0003	No HRP	0.00001	-	0.00001

Poultry fats	Poultry fats	27	0.001	1.1 (90)	0.00003	0.029	0.00003
Poultry, edible offal of	Chicken, offal of	4	0.10	2.7 (97.5)	0.0004	0.011	0.0004
	Duck, offal of	4	0.004	2.7 (97.5)	0.00002	0.011	0.00002
	Goose, offal of	4	0.008	1.0 (90)	0.00003	0.004	0.00003
	Poultry offals, unprocessed	4	0.017	2.0 (97.5)	0.0001	0.008	0.0001
Eggs	Chicken eggs	10	0.46	3.1 (97.5)	0.005	0.031	0.005
	Duck eggs	10	0.016	2.7 (97.5)	0.0002	0.027	0.0002
Plant products	Cucumber	20	0.33	4.6 (97.5)	0.007	0.092	0.007
	Melons, except watermelon	20	0.21	15.8 (97.5)	0.004	0.32	0.004
	Peppers, sweet	150	0.088	0.68 (97.5)	0.013	0.10	0.013
	Potato	10	1.7	6.7 (97.5)	0.017	0.067	0.017
	Tomato	80	1.2	10.6 (97.5)	0.093	0.85	0.093
	Tomato juice	14	0.12	6.9 (97.5)	0.002	0.097	0.002
	Tomato sauce	78	0.022	2.1 (97.5)	0.002	0.17	0.002
Fish and seafood	Salmon, Pacific	425	0.18	2.6 (97.5)	0.076	1.1	0.076
	Trout	348	0.0003	No HRP	0.0001	-	0.0001
						TOTAL	1.8
						L	
						%	9.0
						ADI	

¹For non-fish food types, median residue concentrations were taken from JMPR (2016)

²highest mean consumption figures based on whole population considered from the available dataset

³highest reliable percentile food consumption figures based on consumers only considered from the available dataset

⁴GECDE is the sum of the highest exposure at the highest reliable percentile of consumption for a food and the mean dietary exposures of the other foods

No HRP: No highest reliable percentile could be defined due to low numbers of consumers

Maximum Residue Limits

In recommending MRLs for lufenuron in salmon and trout fillet the Committee considered the following factors:

- An ADI for lufenuron of 0-0.02 mg/kg bw was established by the Committee.
- An ARfD was considered unnecessary.
- The Committee considered that for lufenuron there are no specific concerns for less-than-lifetime exposure.
- Lufenuron is used as both a pesticide and a veterinary drug.
- Lufenuron is authorised for use in salmon in one Member State. The maximum recommended dose is 5 mg/kg per day for 7 consecutive days, administered through medicated feed. The withdrawal period is 2050 degree-days.
- Lufenuron is the marker residue in fillet.
- The ratio of the concentration of marker residue to the concentration of total residue of 1.0 was calculated.
 - Residue data for salmon and trout were provided using a validated analytical method to quantify lufenuron in fillet.
 - A validated analytical method (UHPLC-MS/MS) for the determination of lufenuron in salmon and trout fillet is available and may be used for monitoring purposes, with the reservation noted above.

An MRL was calculated on the basis of the upper limit of the one-sided 95 % confidence interval over the 95th percentile of total residue concentrations (95/95 UTL) in salmon fillet derived from the data provided, in accordance with Good Veterinary Practice and a withdrawal period of 2050 degree-days.

The Committee recommended an MRL of 1350 µg/kg for lufenuron in salmon fillet.

For trout, using the same approach, the Committee could recommend an identical MRL should lufenuron be approved for use in trout. This MRL is compatible with a withdrawal period of 2643 degree-days based on the residue data provided.

The Committee could not extrapolate the MRL to other fish species or to fin fish in general considering that:

- (i) lufenuron is a lipophilic drug and its concentration is higher in fatty tissues, the fat content in fish depends on the species and growing conditions,
- (ii) the decrease in concentration of lufenuron in studies in Atlantic salmon and in rainbow trout is dependent on the time after the last drug administration as well as the increase in body weight, both of which are dependent on the water temperature,
- (iii) no depletion data were provided for species other than salmonids.

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 of 0.02 mg/kg bw per day. Salmon was the major contributor to chronic dietary exposure. It should be noted that no reliable high percentile consumption value was available for trout.

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Monepantel

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Addendum to the monographs prepared by the 75th and 78th meetings of the Committee and published in the FAO JECFA Monographs 12 and 15.

Identity

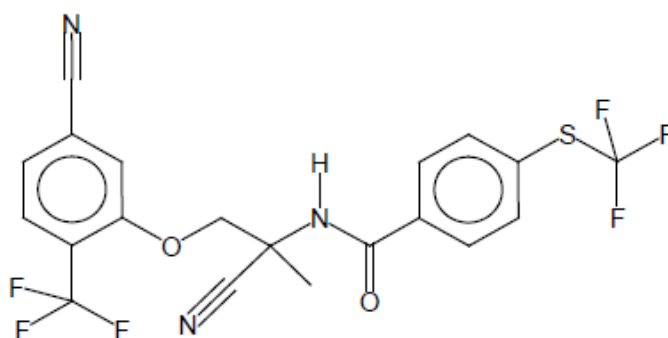
International Non-proprietary Names (INN): Monepantel

Synonyms: N-[2-(5-cyano-2-trifluoromethyl-phenyloxy)-1-(S)-1-cyano-1-methyl-ethyl]-4-trifluoromethylthio-benzoic amide, Zolvix

IUPAC Name: N-[(1S)-1-Cyano-2-(5-cyano-2-trifluoromethyl-phenoxy)-1-methyl-ethyl]-4-trifluoromethylsulfanyl-benzamide

Chemical abstract Service No.: 887148-69-8

Structural formula:



Molecular formula: C₂₀H₁₃F₆N₃O₂S

Molecular weight: 473.4

Other information on identity and properties

Pure active ingredient: AHC 2102225, the active S-enantiomer of NG-96 (the racemic mixture of S- and R-enantiomers)

Appearance: White powder

Impurities: AHC-2155367 = N-[2-(5-cyano-2-trifluoromethyl-phenyloxy)-1-(S)-1-cyano-1-methylethyl]-4-chloro-benzoic amide, residual solvents etc, each specified at <0.5 %

Melting point: 125 °C (polymorphic form A); 142 -149 °C (polymorphic form B)

Solubility: water: 0.1 mg/L at 20 °C

dichloromethane: 175 g/L

ethanol: 60.7 g/L

n-octanol: 7.3 g/L

propylene glycol: 6.9 g/L

polyethylene glycol: 156.1 g/L

Log Ko/w or Partition Coefficient: Octanol/water partition coefficient: log Pow = 3.0 (shake flask method, pH 7, at 20 °C)

pH: 6.2-6.3 (suspension in water)

Optical rotation: $[\alpha]_{580\text{nm}}$ -32° (methanol)

UV_{max}: Note provided

Stability: Store at room temperature; protect from light

Background

Monepantel, an amino-acetonitrile derivative anthelmintic, was reviewed previously by the Committee at its seventy-fifth meeting (FAO, 2011). The Committee established an ADI of 0-20 µg/kg bw, corresponding to an upper bound of acceptable intake of 1200 µg/day for a 60 kg person. Used as an oral drench to control gastrointestinal nematodes (roundworms) in sheep, the Committee recommended MRLs, determined as monepantel sulphone, in sheep tissue of 300 µg/kg in muscle, 700 µg/kg in kidney, 3000 µg/kg in liver and 5500 µg/kg in fat. Because sufficient data were available to calculate median residue values, the EDI approach was used. Using the model diet and marker to total residue ratio of 1 for muscle and 0.66 for fat, liver and kidney, and after applying a correction factor of 0.94 to account for the mass difference between monepantel sulphone (the marker residue) and monepantel, the EDI calculated was 201 µg/person per day, which represents 17 % of the upper bound of the ADI.

At the Twentieth of the Codex Committee on Residue of Veterinary Drugs in Food (CCRVDF), concerns were raised that the recommended MRLs were significantly lower than those already established in some countries and could create trade problems (FAO/WHO, 2012). It also was noted that the recommended MRLs were not consistent with the withdrawal times in some countries. The CCRVDF discussed higher MRLs, recognising that it was within the purview of the Codex Committee, as risk managers, to modify the MRLs recommended by JECFA. Some Delegations did not consider advancing higher MRLs appropriate without an evaluation of their safety by JECFA, in recognition of JECFA's role as risk assessor for Codex. The

CCRVDF agreed to request that JECFA evaluate the safety of the proposed higher MRLs in light of the information provided by the Committee. Specifically, JECFA was asked to consider if higher MRLs (Muscle, 700 µg/kg; Liver, 5000 µg/kg; Kidney, 2000 µg/kg; Fat, 7000 µg/kg) are compatible with the ADI and consistent with the JECFA process for the derivation of MRLs.

Monepantel was subsequently reviewed by the Committee at its seventy-eight meeting (FAO, 2013), which confirmed the ADI of 0–20 µg/kg bw. The Committee recommended revised MRLs in sheep tissue of 500 µg/kg in muscle, 1700 µg/kg in kidney, 7000 µg/kg in liver and 13000 µg/kg in fat. Again, using the model diet and marker to total residue ratio of 1 for muscle and 0.66 for fat, liver and kidney, and after applying a correction factor of 0.94 to account for the mass difference between monepantel sulphone (the marker residue) and monepantel, the revised EDI calculated was 443 µg/person per day, which represents 37 % of the upper bound of the ADI.

Monepantel was added to the priority list at the 23rd meeting of the CCRVDF (Houston, TX, 2016) with the request to recommend MRLs for cattle tissues.

Residues in food and their evaluation

Conditions of use

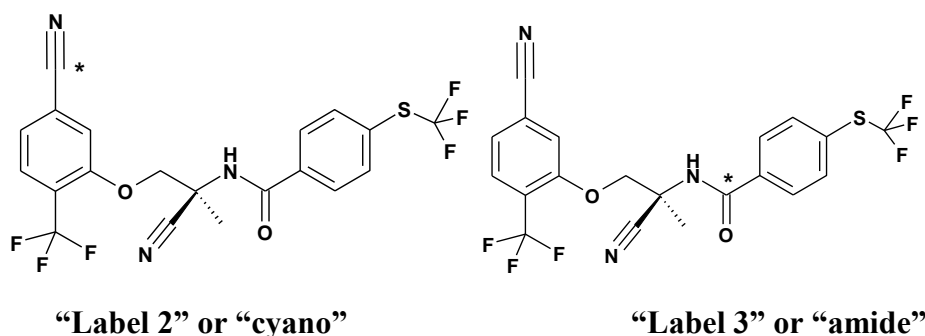
Monepantel is indicated for the treatment of roundworms in cattle. The product, formulated as a solution containing 25 mg monepantel per mL, has recently been approved for use in cattle in New Zealand and is marketed for use in sheep in many countries. For cattle, the EU has established an ADI of 0.03 mg/kg bw. The EU recommended MRLs are 7000, 2000, 1000 and 300 µg/kg for fat, liver, kidney and muscle, respectively (EMA, 2016). The same MRLs have been established in Australia. The assigned withdrawal period in New Zealand for cattle is 5 days. A milk discard time is assigned as part of the registration; however, a milk MRL is not requested from the JECFA and no milk residue data are provided for evaluation.

Dosage

Monepantel is administered to cattle orally, as a drench, at a minimum dose rate of 2.5 mg/kg bw and a maximum dose of 3.7 mg/kg bw. Up to three doses per season can be applied with a minimum retreatment interval of 21 days.

Pharmacokinetics and metabolism

For the pharmacokinetic and metabolism studies, monepantel was radiolabelled at either “label 2” or “cyano” or “label 3” or “amide” (Figure 1).

Figure 1. Positions of labelled [^{14}C]-monepantel

Pharmacokinetics in laboratory animals

The pharmacokinetics of monepantel in laboratory animals were evaluated at the 75th meeting of the Committee (WHO, 2012).

Pharmacokinetics in Food-producing Animals

Cattle

A GLP-compliant ADME study was conducted with [^{14}C]-monepantel (3.75 mg/kg bw) in a placebo base (Vance, 2014). The test material was determined to have a radiopurity of more than 99 % using two distinct chromatographic systems. Monepantel was administered orally once to twelve male and female Aberdeen/Angus cross beef cattle (Table 1). The monepantel was labelled at the amide carbon adjacent to the phenyl ring (“label 3” in Figure 1). Animal 11 was overdosed by 25 % due to complications with gavage dosing, and was slaughtered early at day 3; no correction for overdosing is made below.

Table 1. Study Group Assignments

Study Group	Animal Number	Sacrifice Time Point
1	11M*, 2F, 3F	Study Day 3
2	4M, 5M, 6F	Study Day 7
3	7M, 8F, 9F	Study Day 14
4	10M, 1M*, 12F	Study Day 21

M = Male, F = Female

Animal 1M originally was assigned to Group 1 but was swapped with Animal 11 on Study Day 0 to become a Group 4 animal due to mistaken dosing of Animal 11.

Blood, edible tissues, faeces, bile and urine were collected for up 21 days post-treatment. Three animals were held in metabolism crates for the first 3 days.

Blood samples (*ca.* 20 mL) were collected from Animal 11M pre-dose and at the following time points post dose: 4 h, 8 h, 12 h, 24 h, 36 h, 48 h and 72 h. Blood samples were collected from Animals 10M and 12F pre-dose and at the following time points post dose: 4 h, 8 h, 12 h, 24 h, 36 h, 48 h, 72 h, 96 h, 168 h, 240 h, 312 h, 408 h and 504 h. Blood samples were collected from Animal 1M at the following time points post dose: 24 h, 36 h, 48 h, 72 h, 96 h, 168h, 240

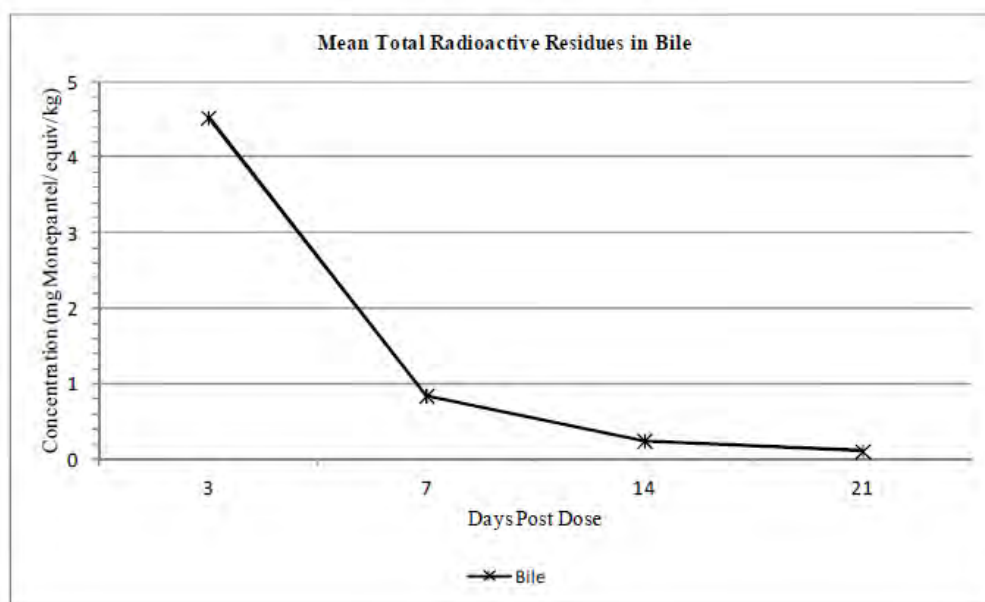
h, 312 h, 408 h and 504 h. Blood samples were collected within ± 10 min of the target time at 4 h, 8 h, 12 h and 24 h time point. The remaining blood samples were collected within ± 30 min of the target time. All blood was sampled from the jugular vein. Immediately following collection, the blood was transferred into 2 x 10 mL tubes containing lithium heparin as anti-coagulant and the samples thoroughly mixed.

Systemic absorption was relatively high, with TRR reaching 216 μg equivalents/kg in blood and 268 μg equivalents/kg plasma at 24 h after dosing (Table 2). Absolute bioavailability was not determined. Initial elimination was rapid with a half-life of about 36 h, but this slowed progressively at later times. Residues were still detectable at the final sampling time point at *ca.* 5 μg equivalents/kg in blood and plasma.

Table 2. Mean TRR concentrations in blood and plasma (mg monepantel equiv/kg)

Time Point (Hours Post Dose)	Concentration (mg monepantel equiv/kg)	
	Blood	Plasma
4	0.073	0.102
8	0.135	0.172
12	0.181	0.230
24	0.216	0.268
36	0.177	0.204
48	0.159	0.175
72	0.103	0.109
96	0.070	0.076
168	0.041	0.045
240	0.027	0.028
312	0.016	0.021
408	0.008	0.009
504	0.005	0.005

Bile TRR was highest at day 3 (~ 4500 $\mu\text{g}/\text{kg}$) but declined progressively to ~ 100 $\mu\text{g}/\text{kg}$ at day 21 (Figure 2).

Figure 2. Mean TRR in Bile

About 21 % of the dose was eliminated in the urine over 3 days. Mean total radioactive residues in urine were similar at one and 2 days post dose (16-18 mg equiv/kg). Subsequently, residues depleted to *ca.* 11 mg equiv/kg by 3 days post dose and to *ca.* 1 mg equiv/kg by 7 days post dose. By 14 and 21 days post dose, concentrations had declined to *ca.* 500 and 200 µg equivalents/kg, respectively (Table 3).

Approximately 36 % of the dose was eliminated in the faeces. In faecal samples, concentrations at one day post dose were approximately half those observed in the urine (*ca.* 9 mg equiv/kg). Concentrations subsequently doubled to *ca.* 17 mg equiv/kg during the following 24-hour period (2 days post dose). By 3 days post dose, concentrations had decreased to similar levels observed at one day post dose (*ca.* 7 mg equiv/kg), before depleting rapidly to *ca.* 600 µg equivalents/kg at 7 days post dose and <100 µg equivalents/kg by 21 days post dose.

Table 3. Mean TRR in Urine and Faeces*

Time Point (Days Post Dose)	Concentration (mg equiv/kg)	
	Urine	Faeces
1	17.725	8.840
2	15.984	16.539
3	11.422	7.081
7	1.327	0.576
14	0.545	0.200
21	0.157	0.084

*Day 1-3 means of animals in metabolism cages; Day 7, 14, and 21 means are from animals at time of slaughter.

Cage rinse was collected at the end of 3 days. Less than 2 % of the administered radioactivity was recovered in the cage rinse.

About 60 % of the dose was recovered in excreta over 3 days, with the remaining material distributed in the tissues (Table 4).

Table 4. Mean Recovery of Total Radioactivity in Urine, Faeces and Cage Rinse*

Time Point (Hours Post Dose)	Total Radioactive Recovery (% of Dose Administered)			
	Urine	Faeces	Cage Rinse	Total
24	9.133	8.575	Not Required	17.708
48	5.894	17.568	Not Required	23.462
72	6.316	10.014	1.771	18.101
Total	21.343	36.157	1.771	59.271

*Means of animals in metabolism cages only

In a second GLP compliant study, the pharmacokinetic profiles of monepantel and monepantel sulphone in blood were investigated in beef cattle dosed three times 21 days apart at 3.75 mg/kg bw monepantel as part of the pivotal cold residue depletion study (Adams and Le, 2014).

Twenty male and female Angus cross cattle, *ca.* 250 kg, were enrolled in the study and allocated to 4 slaughter groups. Animals were maintained on pasture for the duration of the study. Blood was collected from one group at regular intervals after each dose, starting at 4 h. Samples were analysed using a validated LC-MS/MS method (Browning, 2014b, 2014c). Animals were slaughtered at 4, 7, 10 and 13 days following the third (final) dose.

Monepantel sulphone (AHC-2144670) was the dominant residue in the blood. Concentrations peaked at 24 h after each dosing and declined thereafter, with a terminal half-life of *ca.* 3 days. Residues at were 4.09-7.22 ng/mL by day 13. Monepantel concentrations peaked at *ca.* 24 h after the first and second dosing with the maximum concentration of monepantel occurring 12 h after the 3rd dosing, and depleted rapidly. Monepantel residues were less than the LOQ (0.25 ng/mL) at day 13 (Tables 5-7; Figures 3 and 4).

The blood profiles of the animals were compared and no accumulation of monepantel and monepantel sulphone in blood was evident following repeated administration of the test item. Overall, the blood profile (measuring individual metabolites) was similar to that observed in the beef ADME study where only TRRs were measured.

Table 5. Concentrations of monepantel and monepantel sulphone in cattle blood (group 4)- 1st Treatment.

Animal no.	Time post 1 st treatment (day)										
	d0.00	0.17	0.33	0.50	1	2	3	4	7	13	
	monepantel (ng/mL)										
229	<LOQ	9.17	12.2	13.4	19.9	7.93	4.60	1.34	0.279	<LOQ	
239	<LOQ	27.5	19.9	20.0	15.6	5.40	2.02	0.810	<LOQ	<LOQ	
242	<LOQ	17.2	20.0	21.1	31.0	11.1	3.26	1.75	0.501	<LOQ	
243	<LOQ	6.91	9.78	12.2	14.1	5.11	1.72	0.742	<LOQ	<LOQ	
245	<LOQ	15.9	18.1	18.0	18.5	8.14	2.62	1.23	<LOQ	<LOQ	
Mean	<LOQ	15.3	16.0	16.9	19.8	7.54	2.84	1.17	<LOQ	<LOQ	
sd	NA	8.07	4.71	3.96	6.66	2.43	1.15	0.413	NA	NA	
		monepantel sulfone (ng/mL)									
229	<LOQ	28.1	49.1	62.5	110	95.4	56.8	40.0	16.4	8.69	
239	<LOQ	97.7	128	133	156	91.3	59.6	39.2	18.5	4.26	
242	<LOQ	59.4	87.0	91.5	146	132	77.3	55.6	29.8	5.50	
243	<LOQ	25.1	47.0	67.3	99.5	70.2	44.1	30.9	12.2	3.01	
245	<LOQ	55.6	80.1	84.7	116	103	68.5	53.5	18.8	3.34	
Mean	<LOQ	53.2	78.2	87.8	126	98.4	61.3	43.8	19.1	4.96	
sd	NA	29.3	33.1	28.0	24.3	22.4	12.5	10.4	6.52	2.30	

^dday -4 pre-treatment

NA = Not available

Table 6. Concentrations of monepantel and monepantel sulphone in cattle blood (group 4)- 2nd Treatment.

Animal no.	Time post 2 nd treatment (day)									
	^e 0.00	0.17	0.33	0.50	1	2	3	4	7	13
	monepantel (ng/mL)									
229	<LOQ	7.97	10.1	13.7	25.4	10.3	3.37	1.95	0.463	<LOQ
239	<LOQ	10.5	16.7	18.5	17.4	5.59	2.51	1.05	<LOQ	<LOQ
242	<LOQ	11.4	12.9	14.1	24.7	14.0	5.40	2.94	0.764	0.529
243	<LOQ	6.70	8.99	13.3	16.0	4.86	1.57	0.718	<LOQ	<LOQ
245	<LOQ	7.72	11.2	12.3	14.6	5.38	2.57	1.19	0.299	<LOQ
Mean	<LOQ	8.86	12.0	14.4	19.6	8.03	3.08	1.57	0.509	<LOQ
sd	NA	1.99	3.01	2.40	5.06	3.99	1.44	0.889	NA	NA
	monepantel sulfone (ng/mL)									
229	1.99	32.8	58.8	81.5	163	135	81.6	66.8	21.3	7.73
239	1.20	56.1	98.6	124	168	113	79.9	50.9	16.0	6.01
242	3.74	38.1	62.2	77.5	135	127	87.5	66.1	32.7	13.2
243	0.60	38.5	68.6	109	169	105	53.9	31.2	11.8	4.52
245	0.68	56.9	98.1	112	164	108	70.4	46.3	18.0	4.64
Mean	1.64	44.5	77.3	101	160	118	74.7	52.3	20.0	7.22
sd	1.30	11.2	19.6	20.3	14.1	12.9	13.1	14.9	7.91	3.59

^e20 days post 1st treatment

NA = Not available

Table 7. Concentrations of monepantel and monepantel sulphone in cattle blood (group 4)- 3rd Treatment.

Animal no.	Time post 3 rd treatment (day)									
	^f 0.00	0.17	0.33	0.50	1	2	3	4	7	13
	monepantel (ng/mL)									
229	<LOQ	11.5	16.8	21.8	22.7	6.07	2.00	0.937	0.396	<LOQ
239	<LOQ	14.5	22.0	24.6	15.2	4.83	1.50	1.11	<LOQ	<LOQ
242	<LOQ	22.1	30.7	32.6	22.5	6.45	2.05	0.953	0.298	<LOQ
243	<LOQ	19.6	24.8	25.0	13.9	3.72	1.06	0.481	<LOQ	<LOQ
245	<LOQ	16.0	23.7	26.7	32.7	5.37	1.80	0.658	<LOQ	<LOQ
Mean	<LOQ	16.7	23.6	26.1	21.4	5.29	1.68	0.828	<LOQ	<LOQ
sd	NA	4.18	5.02	4.02	7.50	1.08	0.409	0.253	NA	NA
	monepantel sulfone (ng/mL)									
229	2.85	40.3	74.0	109	155	94.3	53.4	36.7	18.5	4.51
239	1.17	47.3	94.7	134	127	74.4	43.1	40.1	10.6	2.89
242	4.53	65.1	109	129	147	94.3	54.7	34.6	17.5	6.51
243	1.25	52.9	92.3	129	134	67.5	33.7	20.8	8.75	3.25
245	1.00	52.5	99.5	138	160	90.1	50.4	27.9	13.0	3.27
Mean	2.16	51.6	93.9	128	145	84.1	47.1	32.0	13.7	4.09
sd	1.52	9.10	12.8	11.2	13.9	12.4	8.72	7.69	4.24	1.49

^f20 days post 2nd treatment

NA = Not available

Figure 3. Mean blood concentrations of monepantel following oral administration of monepantel three times 21 days apart to beef cattle

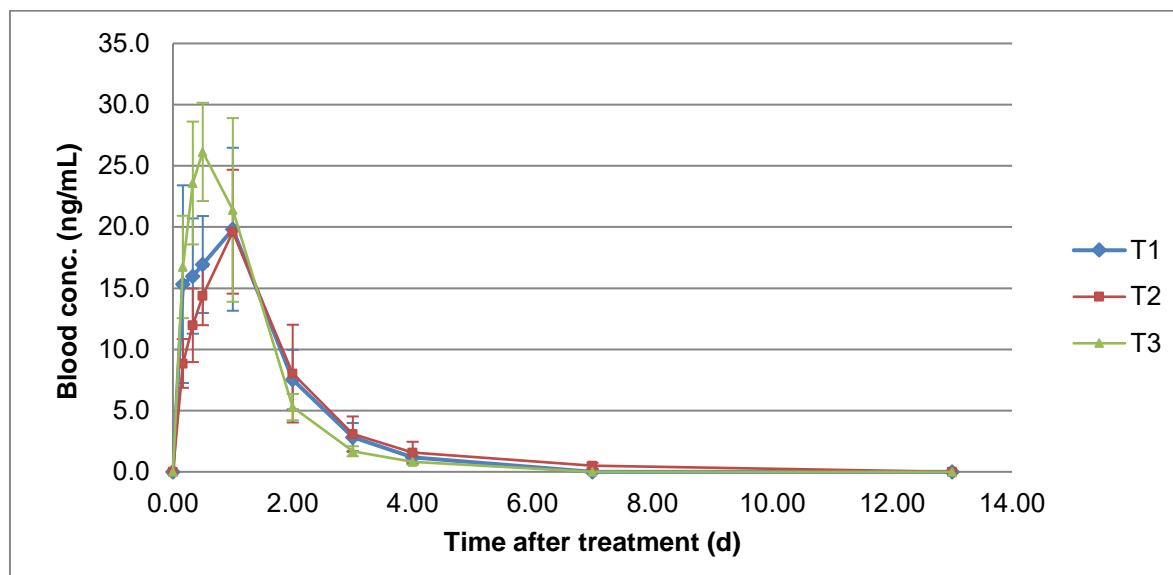
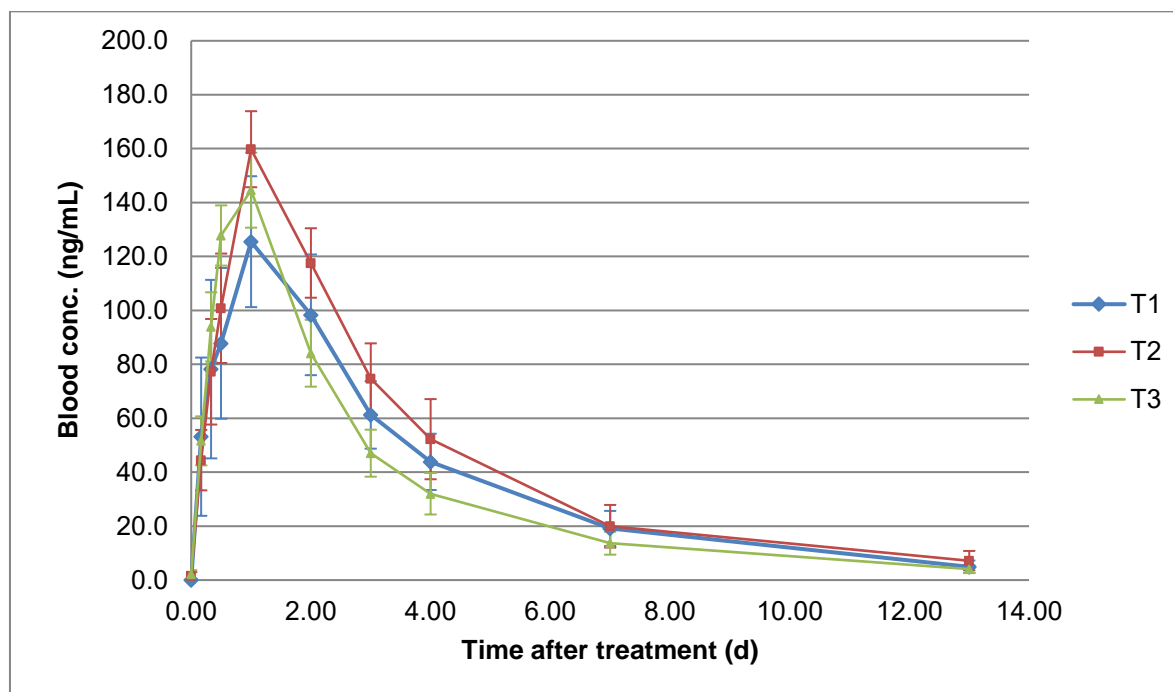


Figure 4. Mean blood concentrations of monepantel sulphone following oral administration of monepantel three times 21 days apart to beef cattle



Sheep

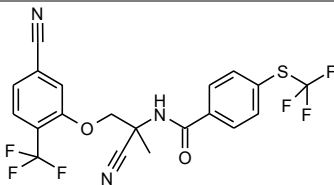
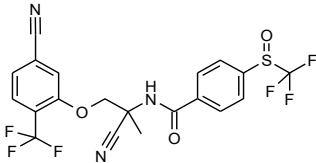
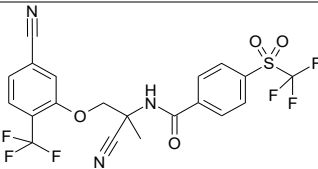
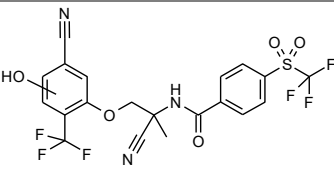
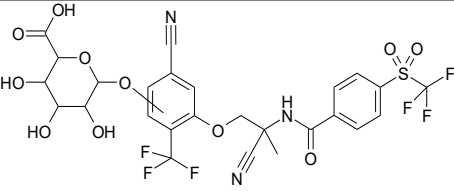
The pharmacokinetic behaviour of monepantel and monepantel sulphone in the blood and plasma of sheep was evaluated at the 75th meeting of the Committee (FAO, 2011; WHO, 2012).

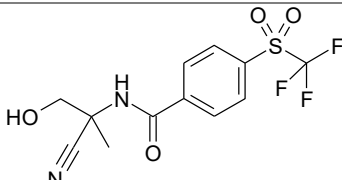
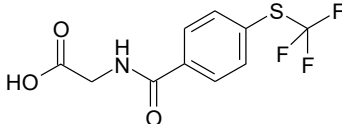
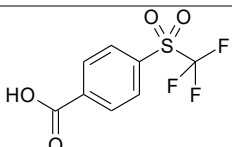
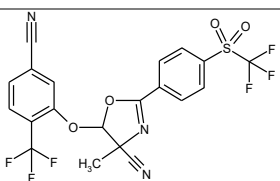
Metabolism in Laboratory Animals

Rats

The ADME of monepantel was evaluated by the 75th meeting of the Committee (FAO, 2011; WHO, 2012). Metabolism of monepantel proceeds by rapid oxidation of the sulphur to the sulphone with slower hydroxylation of the phenoxy ring. The resulting metabolites are nonpolar. There are multiple cleavages of the amino-acetonitrile bridge to polar metabolites. Elimination is via the urine and faeces of the sulphation or glucuronidation of these metabolites. A minor metabolite, AHC-2197876, is found in fat and, to a lesser extent, muscle. The metabolic pathways in the rat are summarized in (Figure 2 in WHO, 2012).

Table 8. Metabolite nomenclature

Metabolite	Code	Description/other names	Structure
Monepantel	AHC-2102225	Parent	
M1		Sulfoxide of parent	
M2	AHC-2144670	Sulphone Monepantel sulphone	
M3		Hydroxylated M2 F24	
M6		Glucuronide of M3	

M9		Cleaved metabolite U15b	
M10		Glycine conjugate of trifluoromethylthiobenzoic acid	
M11		Cleaved metabolite U17b	
G32	AHC- 2197876	Cyclized metabolite	

Metabolism in Food Producing Animals

Cattle

Samples of edible tissues, blood, bile and excreta from the GLP compliant ADME study (Vance, 2014) were investigated for extractability of residues, and metabolite profiles. Due to low TRR, kidney at day 21 and muscle at days 7 to 21 were not investigated (Table 9).

Radioactivity was extracted readily from fat, kidney and muscle, with simple solvent extraction. Less than 5 % of TRR was unextractable.

Liver residues proved more difficult to extract, especially with increasing time after dose administration. Unextracted residues increased from 37 % on Day 3 to 75 % on Day 14. Simple solvent extraction could remove about half of the TRR at day 3 and this dropped to *ca.* 13 % on day 21. Further extractions with more polar solvents, water and ammonia did not improve extraction significantly. Extracted day 21 TRR was too low to proceed with metabolite profiling.

Table 9. Metabolites in edible tissues of cattle

Tissue	Peak	%TRR (mg/kg)			
		Day 3	Day 7	Day 14	Day 21
Liver	Parent	0.5 (0.012)	ND	ND	-
	AHC 2144670	36.5 (0.855)	4.5 (0.074)	6.4 (0.042)	-
	Unknowns (by decreasing polarity)	13.7 (0.320)	1.1 (0.018)	1.8 (0.011)	-
		5.8 (0.136)	2.1 (0.034) 1.1 (0.018)	0.7 (0.005) 0.6 (0.004)	
Kidney	Parent	5.2 (0.068)	7.5 (0.040)	ND	-
	AHC 2144670	67.9 (0.888)	65.8 (0.350)	94.9 (0.214)	-
	Unknowns (by decreasing polarity)	4.8 (0.063)	12.0 (0.064)	ND	-
		11.6 (0.152) 7.2 (0.094)			
Muscle	Parent	ND	-	-	-
	AHC 2144670	76.3 (0.152)	-	-	-
	Unknown	10.4 (0.021)	-	-	-
Fat	Parent	9.5 (0.627)	4.8 (0.115)	10.0 (0.089)	ND
	AHC 2144670	88.2 (5.834)	73.5 (1.762)	82.0 (0.730)	77.7 (0.252)
	AHC 2197876	0.8 (0.051)	1.4 (0.033)	5.8 (0.051)	9.3 (0.030)

ND = Not Detected - = Not Analyzed

The major component present in all tissues cochromatographed with AHC 2144670, monepantel sulphone. The parent molecule, monepantel, was a minor constituent, present only at the early timepoints in fat, liver and kidney, and was not detected in the muscle. Minor unknown components of high polarity were detected in the liver (n=3), kidney (n =3) and muscle (n=1).

In liver, two minor polar metabolites (referred to as L1 and L2, Figures 5 and 6) were observed at days 3–14 (Strathdee, 2016). High resolution MS (accurate mass determination and fragmentation patterns via MS/MS and MS3) and radio-HPLC detection were used to identify metabolite L1 as the cleaved metabolite M9 and metabolite L2 as a further cleaved M11 (Table 8 above).

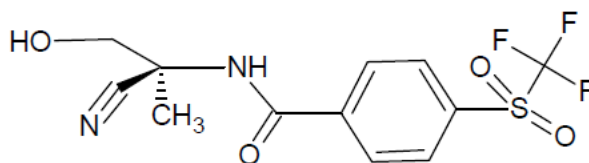
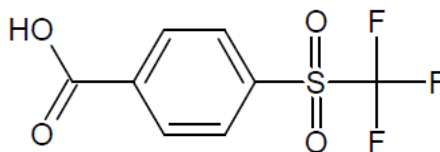
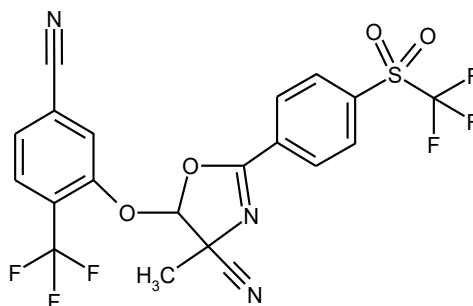
Figure 6. Postulated structure of Metabolite L1

Figure 6. Postulated structure of Metabolite L2

Kidney profiles were similar to those in liver, including the presence of small amounts of L1 and L2. A third polar metabolite was observed only at day 3 and it was <5 % of TRR.

A component, detected only in the fat, cochromatographed with AHC 2197876 and was determined to be a cyclised metabolite (Figure 7).

Figure 7. Postulated structure of cyclised metabolite in fat

The small amount of TRR in muscle limited profiling to the Day 3 samples. Only a trace of L1 was detected.

The major metabolite in blood was monepantel sulphone, AHC 2144670. There was a small amount of parent monepantel and several minor metabolites (Table 10).

Table 10. Metabolic profile in Day 3 cattle blood

Metabolite	Retention Time (min)	%TRR	mg/kg
Combined and Concentrated			
-	6.63	3.9	0.004
-	7.88	2.9	0.003
-	10.63	10.3	0.011
-	13.13	2.6	0.003
AHC 2144670	24.88	13.7	0.014
Monepantel	25.63	1.8	0.002
Total characterised/ Identified		35.2	0.037

The metabolic profile in bile was complex (Table 11). Non-polar metabolites were readily identified by co-chromatography with authentic standards. In addition to parent monepantel, there was a trace of monepantel sulphone, which was confirmed by LC-MS/MS, as part of the analytical method validation used to establish the MR/TRR ratio (Browning, 2014a). Additionally, there was a large peak of intermediate polarity, a large polar peak with retention time corresponding to L1 and a cluster of more polar peaks.

Table 11. Metabolic profile in Day 3 cattle bile

Component	Retention Time (min)	%TRR (%)
Unknowns	0.63	0.7
	1.88	0.6
	3.63	2.2
	5.63	26.4
	9.88	18.1
	10.88	2.1
	12.38	1.6
	13.38	0.6
	14.13	0.6
	14.88	1.3
	15.88	29.8
	17.38	7.8
	20.38	0.6
	22.88	0.5
AHC 2144670	24.38	4.3
Monepantel	26.88	0.6
Unknowns	32.13	0.3
	33.88	1.0
Total Characterised by HPLC		99.1
Total Extracted		99.3
Unextracted		0.7
Total		100

In urine, there was one polar metabolite and four minor polar peaks (Table 12). No parent monepantel, monepantel sulphone (AHC-2144670) or hydroxylated monepantel sulphone was detected.

Table 12. Metabolic profile in Day 2 cattle urine

Component	Retention Time (min)	%TRR (%)
Unknowns	8.45	8.5
	10.63	9.7
	11.72	5.5
	13.25	66.3
	17.77	6.6
Total Characterised by HPLC		96.6
Total Extracted		96.7
Unextracted		3.3
Total		100

Faecal residues were easily extracted with simple solvent extraction (>80 %) (Table 13).

Table 13. Metabolites in Faeces

Extract/Peak	%TRR in Faeces				
	Day 1	Day 2	Day 3	Day 7	Day 14
Parent	43.9	25.9	16.3	3.8	ND
AHC 2144670	10.4	9.4	7.0	28.6	28.5
Unknowns	37.5	53.1	55.6	41.8	36.9

ND = Not Detected

Sheep

The metabolism of monepantel in tissues of sheep was evaluated at the 75th meeting of the Committee (FAO, 2011).

Comparative metabolism

A non-GLP in vitro metabolism study was conducted to investigate the metabolism of monepantel in intact rat, cattle and sheep hepatocytes (Anderson *et.al.*, 2016). Both ¹⁴C-labeled analogues of monepantel (Figure 1) were used in the study. The activity of hepatocytes was evaluated using verapamil. The activity of rat and bovine hepatocytes was consistent with historical controls. Sheep hepatocytes were about 30 % less active than hepatocytes from cattle and rats but there were no historical data for comparison.

Following MeOH quenching at 0, 1, 2, and 4 h, incubations were profiled by HPLC with radio-detection. Metabolites in these extracts were identified by LC/MS techniques. The protein pellet was extracted further with various solvents to determine the extent of covalent binding.

Covalent binding was minimal, but occurred in all three species and for both label types. Binding was highest in bovine hepatocytes and lowest in ovine hepatocytes (bovine > rat >

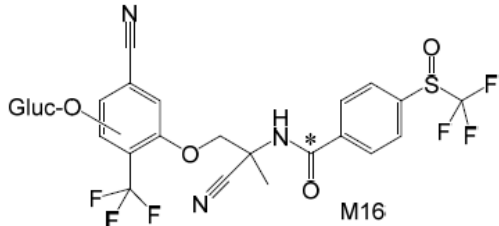
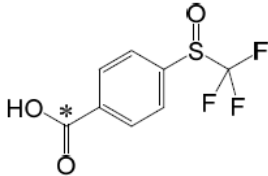
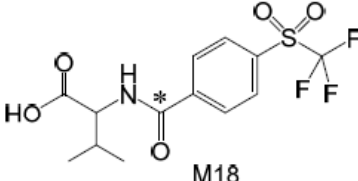
sheep). The lower binding in sheep hepatocytes was attributed to the lower metabolic turnover. The radioactivity recovered in the methanol extracts further indirectly supports the conclusion of minimal binding for monepantel and its metabolites, with between 96 to 105% recovered at 1, 2, and 4 h.

Metabolism was greatest with rat hepatocytes. Less than 1 % monepantel remained after 2 h incubation. With bovine and sheep hepatocytes, 13 % and 66 % of monepantel remained after 4 h.

In incubations of rat hepatocytes, the predominant metabolites were M2 and M6. M2 peaked at 1 hour, accounting for 45 % of the extracted radioactivity. M6 peaked at 2 h, accounting for 49-52 % of the extracted radioactivity. In incubations of bovine hepatocytes, the major metabolites were M1, M2 and M10, accounting for 16 %, 27 % and 16 % respectively of extracted radioactivity, in the 4 hour sample. In incubations of sheep hepatocytes, the major metabolites were M1, M2 and M10, accounting for 10 %, 4 %, and 9 % respectively of extracted radioactivity, in the 4 hour sample.

Three new metabolites were observed in the in vitro study (Table 14). In rat hepatocytes, a new metabolite (M16) was observed at <2 % of applied radioactivity. In bovine hepatocytes, two new minor metabolites (M17 and M18) were observed at <5 % or less of applied radioactivity. Fewer metabolites were observed in incubations with sheep hepatocytes and these were limited to the sulphur oxidation products and only one cleavage product. No new metabolites were observed.

Table 14. New metabolites identified in the in vitro hepatocyte study

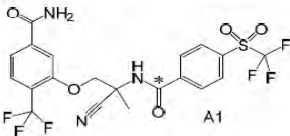
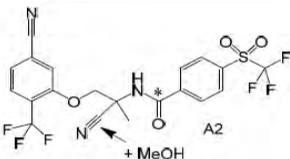
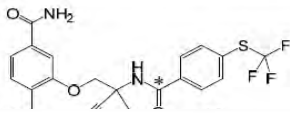
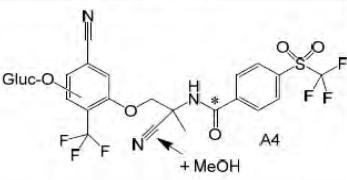
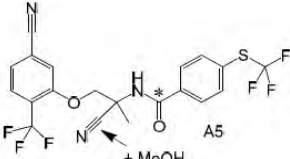
Metabolite	Description/other names	Structure*
M16	Glucuronide of hydroxylated M1	
M17	Trifluoromethylsulfinylbenzoic acid	
M18	Valine conjugate of M11	

* indicates position of ¹⁴C label

Incubations with bovine hepatocytes metabolized monepantel in a similar manner to incubations with rat hepatocytes (Figures 8-10). Although two new metabolites were observed for cattle but not for rats, these were at very low concentrations. Additionally, because the valine conjugate (M18) potentially can be hydrolysed back to M11 by stomach juice and the M1 cleavage product (M17) could be oxidized to M11, these are likely of no toxicological significance (Anderson *et.al.*, 2016).

In addition to the reported metabolites, five chemical adducts (A1 to A5) also were observed in this study (Table 15). Adducts observed in the 0-hour samples were the result of addition at the aromatic or aliphatic cyano moieties of monepantel. Adducts observed in the incubated samples were the result of addition at the cyano moiety of either M2 or M6. Adducts were observed only for the predominant peaks in the metabolic profiles. Because these adducts did not appear to grow over the time course of the incubations, they were determined to be chemical artefacts formed during the workup procedure (Anderson *et.al.*, 2016).

Table 15. Water and MeOH adducts identified in the in vitro hepatocyte study

Peak ¹	[M-H] ⁻	Proposed Metabolite Identification	Structure ²	Rat	Bovine	Sheep
A1 ¹	524	M2 + H ₂ O to form amide on aromatic cyano group		X	X	MS
A2 ¹	538	M2 + MeOH to form N-methylamide on aliphatic cyano group		X	X	X
A3 ^{1,5}	492	P + H ₂ O to form amide on aromatic cyano group		X	X	X
Peak ¹	[M-H] ⁻	Proposed Metabolite Identification	Structure ²	Rat	Bovine	Sheep
A4 ¹	730	M6 + MeOH to form N-methylamide on aliphatic cyano group		X		
A5 ^{1,5}	506	P + MeOH to form N-methylamide on aliphatic cyano group		X	X	X

“X” indicates the metabolite was quantified in the radioprofile of at least one sample. “MS” indicates the metabolite was not quantified in a radioprofile but was detected by mass spectrometry, hence present only at low concentrations.

- ¹ Metabolites were labelled following the nomenclature from the in vivo rat metabolism study, RCC study number A89493 (Gassen 2007 in WHO, 2012), where metabolites M1 through M15 were previously reported. Metabolites M16 through M18 and chemical adducts A1 through A5 were observed for the first time in this study.
- ² The asterisk denotes the site of the ¹⁴C label position 3 and indicates that the monitored M-H anion included the ¹⁴C isotope. Due to lower specific activity, for compounds with radiolabel position 2, the ¹²C isotope was used.
- ³ Metabolites M1, M2, M5, M9, and M11 were previously reported in the in vivo sheep metabolism study (Jung *et.al.*, 2007 in WHO, 2012).
- ⁴ The identity of metabolite M5 was previously matched to standard AHC2166637 (Gassen, 2007, and Jung *et.al.*, 2007, in WHO, 2012).
- ⁵ Chemical adducts A3 and A5 were observed in the 0-hour rat, bovine, and/or sheep hepatocyte samples.

Figure 8. Metabolic Pathways and Metabolites of Monepantel in Rat (R), Bovine (B) and Sheep (S) hepatocytes

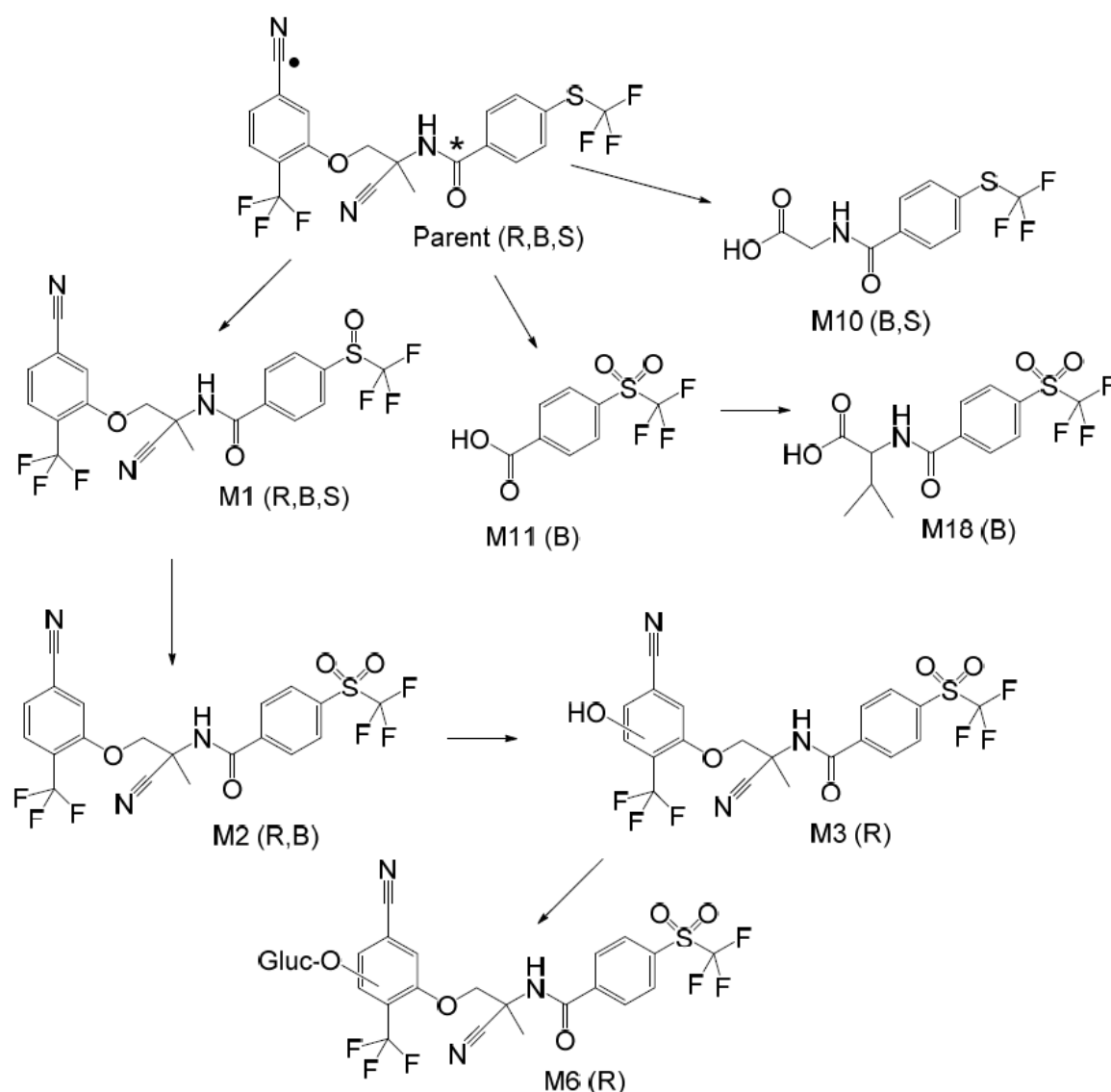
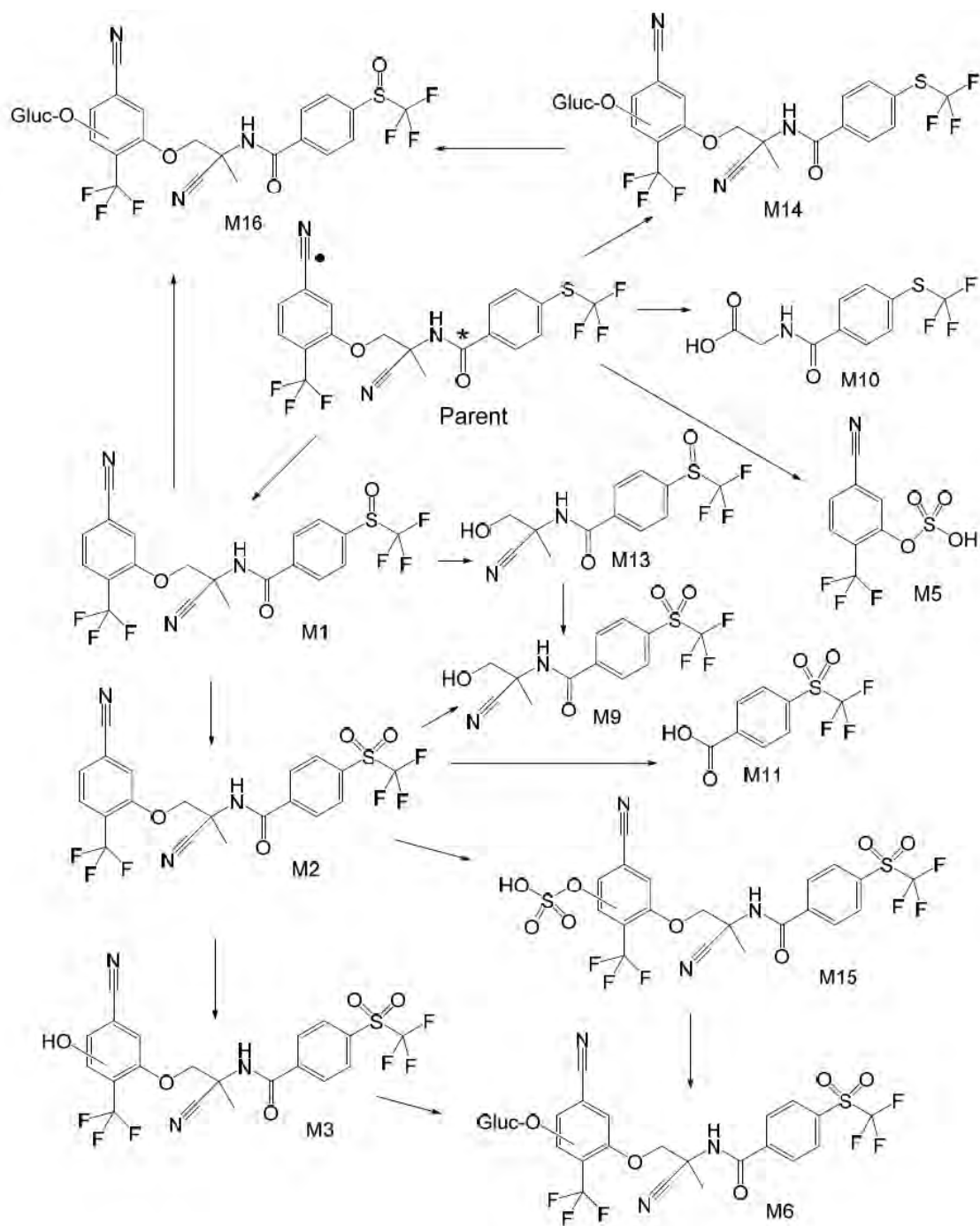
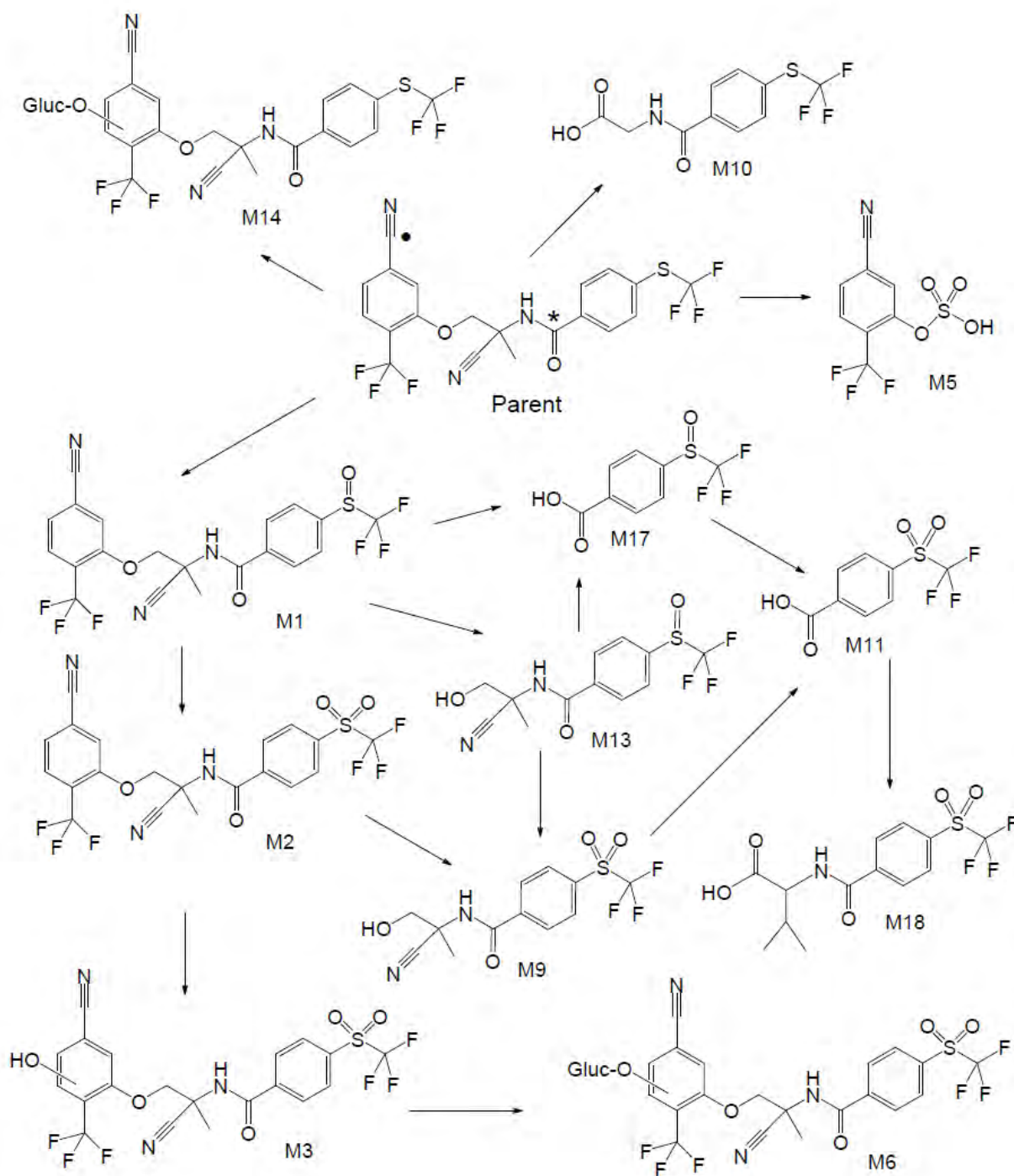


Figure 9. Proposed metabolic scheme for radiolabelled monepantel in rat hepatocytes

• Dot indicates ¹⁴C-radiolabel position 2

* Asterisk indicates ¹⁴C-radiolabel position 3

Figure 10. Proposed metabolic scheme for radiolabelled monepantel in cattle hepatocytes

• Dot indicates ¹⁴C-radiolabel position 2

* Asterisk indicates ¹⁴C-radiolabel position 3

The results of the in vitro study (Anderson *et.al.*, 2016) are consistent with those in the in vivo study (Vance, 2014). The observed metabolites have previously been detected in in vivo studies in rat, and sheep (FAO, 2011; WHO, 2012; Table 8, above, and Table 16).

Table 16. Comparative metabolism in rats and sheep from WHO, 2012

Tissue	Rats (% activity in matrix)	Sheep (% activity in matrix)
Blood	Parent (23%), AHC 2144670 (45%)	AHC 2144670 (100%)
Muscle	Parent (24%), AHC 2144670 (62%)	Parent (5.2%), AHC 2144670 (93%), Mu1 (2%)
Liver	Parent (19%), AHC 2144670 (41%), M3 (14%), M9 (6.8%), L21 (4.2%), M6 (1.1%)	Parent (1.1%), AHC 2144670 (92%), M9 (4.5%), L21 (1.4%)
Fat	Parent (44%), AHC 2144670 (54%)	Parent (14%), AHC 2144670 (77%), Fa1 (7.4%)
Kidney	Parent (20%), AHC 2144670 (48%), K3 (2%)	Parent (5.8%), AHC 2144670 (73%), K3 (22%)

Bovine liver residues are less extractable than sheep or rat liver residues. Bound residues are found in all three species. The higher bound residues in cattle liver are consistent with the shorter terminal half-life of monepantel sulphone in the blood of cattle, *ca.* 3 days following 3 oral doses of monepantel, compared to the terminal half-life of monepantel sulphone in the blood of sheep, nearly 6 days following a single IV dose of monepantel or more than 4 days following a single IV dose of monepantel sulphone (Karadzovska *et.al.*, 2008).

The in vivo metabolism study demonstrated that the cleavage pathway is more prominent in cattle than in other species. The hydrolysis of the amide bond of intact monepantel metabolites forms free amines and carboxylic acids, both of which are candidates for peptide bond formation with free carboxylic acid and free amine groups of endogenous proteins. Indeed, a valine conjugate of M11 was observed demonstrating conjugation to available amino acids, and a glycine conjugate (M10) of trifluoromethylthiobenzoic acid was an in-vitro bovine metabolite.

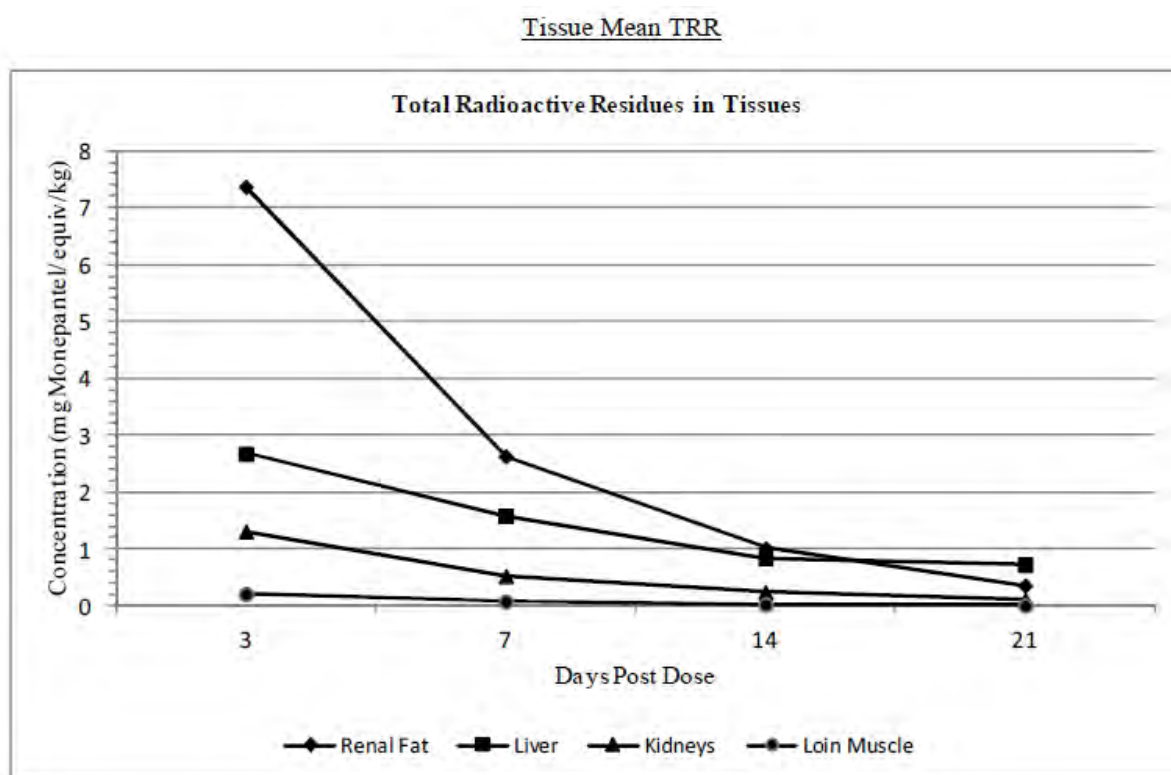
Tissue residue depletion studies

Radiolabelled residue depletion studies

Cattle

Selected samples of edible tissues from the GLP compliant ADME study (Vance, 2014) were investigated for TRR. Monepantel was labelled at the amide carbon adjacent to the phenyl ring ("label 3" in Figure 1). TRRs were determined after a single oral dose at 3.75 mg/kg using combustion and liquid scintillation counting (LSC). Residue data were subjected to log-linear regression analyses (Strehlau, 2014), and log-linearity was observed for fat, kidney and muscle, but not for liver. The calculated half-lives of TRR were 4-5 days for fat, kidney and muscle but about 10 days for liver, consistent with presence of bound residues. Of the edible tissues, kidney samples at day 21 and muscle samples at days 7 to 21 were not investigated due to low TRR.

Figure 11. Depletion profiles of radiolabelled residues in cattle tissues



Edible tissue TRRs are summarized in Table 17-20 and Figure 11. The order of residues in tissues is fat > liver > kidney > muscle, indicative of lipophilic substances, and is the same as seen for sheep. Total residues were more persistent in cattle liver than in the other tissues and more than in sheep liver (FAO, 2011).

Table 17. TRR ($\mu\text{g equiv/kg}$; mean \pm SD) in edible tissue of beef cattle

Day	Fat	Liver	Kidney	Muscle
3	7373 \pm 1164	2677 \pm 256	1315 \pm 328	201 \pm 26
7	2636 \pm 466	1587 \pm 136	531 \pm 86	80 \pm 39
14	1019 \pm 107	843 \pm 42	246 \pm 25	28 \pm 10
21	362 \pm 182	725 \pm 177	111 \pm 51	11 \pm 4

Table 18. TRR as % of administered dose

Group	Euthanasia Days Post Dose	Total Radioactive Recovery (% of Dose Administered)	
		Liver	Kidney
1	3	0.982	0.075
2	7	0.613	0.037
3	14	0.321	0.017
4	21	0.281	0.007

Table 19. Metabolites as % TRR

Tissue	Peak	%TRR (mg/kg)			
		Day 3	Day 7	Day 14	Day 21
Liver	Parent	0.5 (0.012)	ND	ND	-
	AHC 2144670	36.5 (0.855)	4.5 (0.074)	6.4 (0.042)	-
	Unknowns (by decreasing polarity)	13.7 (0.320) 5.8 (0.136)	1.1 (0.018) 2.1 (0.034) 1.1 (0.018)	1.8 (0.011) 0.7 (0.005) 0.6 (0.004)	-
Kidney	Parent	5.2 (0.068)	7.5 (0.040)	ND	-
	AHC 2144670	67.9 (0.888)	65.8 (0.350)	94.9 (0.214)	-
	Unknowns (by decreasing polarity)	4.8 (0.063) 11.6 (0.152) 7.2 (0.094)	12.0 (0.064)	ND	-
Muscle	Parent	ND	-	-	-
	AHC 2144670	76.3 (0.152)	-	-	-
	Unknown	10.4 (0.021)	-	-	-
Fat	Parent	9.5 (0.627)	4.8 (0.115)	10.0 (0.089)	ND
	AHC 2144670	88.2 (5.834)	73.5 (1.762)	82.0 (0.730)	77.7 (0.252)
	AHC 2197876	0.8 (0.051)	1.4 (0.033)	5.8 (0.051)	9.3 (0.030)

ND = Not Detected - = Not Analyzed

Table 20. Ratio of MR to TRR in bovine tissues

Time (day)	Animal	Fat		Liver		Kidney		Muscle		
		TRR	Sulphone 1	%	TRR	Sulphone 1	%	TRR	Sulphone 1	%
3	11M	8472	7393	MR/ TRR	(µg/kg)	(µg /kg)	MR/ TRR	(µg/kg)	(µg/ kg)	MR/ TRR
	2F	7494	5862	87	2935	860	29	1520	812	53
	3F	6153	5410	78	2673	823	31	1488	753	51
	Mean	7373	6222	88	2423	732	30	937	561	60
7	4M	2519	2262	84	2677	805	30	1315	709	55
	5M	2240	1916	90	1576	404	26	623	327	53
	6F	3149	2801	86	1729	305	18	518	301	58
	Mean	2636	2326	88	1587	312	16	452	340	75
14	7M	992	769	89	1457	226	20	531	323	62
	8F	928	769	78	892	67	8			
	9F	1136	855	83	817	104	13			
	Mean	1019	798	75	820	154	19			
21	10M	573	364	79	843	108	13			
	1F	254	179	64	921	89	10			
	12F	260	199	70	677	22	3			
	Mean	362	247	77	578	22	4			

¹ Raw sulphone result adjusted for MW (473/505) and for unmeasured [¹⁴C] monepantel sulphone (2.7 % of TRR: ¹⁴C monepantel (12.54 g) in 467.976 g placebo formulation) as only [¹²C]-monepantel sulphone was measured by LC-MS/MS

Blank entries indicate no analysis for monepantel sulphone

As the major residue is monepantel sulphone (AHC-2144670), it is the marker residue identified for monepantel in cattle tissues. It also is the marker identified previously for sheep tissues (FAO; 2011).

Concentrations of the monepantel sulphone were determined as part of the method validation study (Browning 2014a) and used to determine the MR to TRR ratio cattle tissues (Table 21).

Table 21. MR to TRR ratios

Day	Fat	Liver	Kidney	Muscle
3	84	30	55	67
7	88	20	62	
14	79	13		
21	70	6		

Lactating dairy cows

No radiolabelled residue data were provided for the use of monepantel in lactating dairy cattle.

Sheep

Radiolabelled residue data for the use of monepantel in sheep were evaluated by the 75th meeting of the Committee (FAO, 2011).

Residue depletion studies with unlabelled drug

Cattle

Two GLP-compliant residue depletion studies were conducted in cattle using unlabelled monepantel.

The first study (Adams and Le, 2014) was conducted using 20, 9-month old crossbred beef cattle. Cattle were treated orally three times at a nominal dose of 3.75 mg monepantel/kg body weight. Doses were administered 21 days apart. Animals were slaughtered 4, 7, 10 and 13 days after the final treatment. Samples of muscle, kidney, liver, renal fat and subcutaneous fat were collected and analysed for monepantel sulphone.

The maximum monepantel sulphone residues were observed in the Day 4 samples: 4110, 4680, 1090, 478 and 231 µg/kg for subcutaneous fat, renal fat, liver, kidney and muscle, respectively. At the final sampling time, maximum residues were 1720, 770, 146, 61.8 and 22.8 µg/kg for subcutaneous fat, renal fat, liver, kidney and muscle, respectively (Tables 22 - 24). While initial residues in renal fat were highest, by the later sampling times residues were highest in subcutaneous fat. The calculated half-life estimates for muscle, kidney, liver, renal and subcutaneous fat were 2.8, 2.7, 2.7, 2.9 and 5.1 days, respectively.

Table 22. Residues of monepantel sulphone in subcutaneous and renal fats of cattle treated three times, 21 days apart, with a nominal dose of 3.75 mg monepantel/kg body weight

Group	Day post 3 rd treatment	Animal no.	Subcutaneous fat(µg/kg)		Renal fat(µg/kg)	
			Uncorrected	Recovery corrected	Uncorrected	Recovery corrected
1	4	250	3480	3497	4600	4623
		237	4110	4131	4630	4653
		238	3660	3678	4680	4704
		228	2690	2704	3920	3940
		246	3030	3045	4270	4291
2	7	240	2020	2030	1820	1829
		247	2730	2744	2940	2955
		232	2850	2864	3090	3106
		241	2080	2090	2440	2452
		249	2140	2151	2410	2422
3	10	233	1270	1276	1130	1136
		231	1340	1347	958	963
		236	1120	1126	1460	1467
		227	977	982	970	975
		230	1320	1327	1140	1146
4	13	245	527	530	346	348
		239	859	863	352	354
		243	1460	1467	759	763
		243	<u>1430</u>	1428	not reanalyzed	not reanalyzed
		243	<u>1310</u>	1308	not reanalyzed	not reanalyzed
		242	1220	1226	770	774
		242	<u>1420</u>	1418	not reanalyzed	not reanalyzed
		242	<u>1550</u>	1547	not reanalyzed	not reanalyzed
		229	1720	1729	538	541
		229	<u>1610</u>	1607	not reanalyzed	not reanalyzed
		229	<u>1720</u>	1717	not reanalyzed	not reanalyzed

Underlined italic values are results of repeated analysis

Table 23. Residues of monepantel sulphone in liver, kidney, and muscle of cattle treated three times, 21 days apart, with a nominal dose of 3.75 mg monepantel/kg body weight

Group	Day post 3 rd treatment	Animal no.	Liver(µg/kg)		Kidney(µg/kg)		Muscle(µg/kg)	
			Uncorrected	Recovery corrected	Uncorrected	Recovery corrected	Uncorrected	Recovery corrected
1	4	250	1070	1119	439	468	96.3	96.8
		237	1010	1056	478	510	93.8	94.3
		238	1090	1140	386	412	113	114
		228	870	910	358	382	231	232
		246	978	1023	380	405	156	157
		240	416	435	193	206	38.6	38.8
		247	666	696	337	359	82.8	83.3
		232	764	799	310	330	92.7	93.2
2	7	241	477	499	237	253	42.5	42.7
		249	462	483	237	253	85.3	85.8
		233	255	267	132	141	66.3	66.7
		231	218	228	100	107	29.9	30.1
		236	222	232	150	160	43.0	43.2
		227	284	297	94.3	101	20.2	20.3
3	10	230	226	236	85.2	90.8	46.4	46.7
		245	64.4	67.3	27.9	29.7	13.0	13.1
		239	87.7	91.7	34.0	36.2	11.0	11.1
4	13	243	88.9	93.0	35.7	38.1	7.99	8.03
		242	146	153	61.8	65.9	22.8	22.9
		229	127	133	55.6	59.3	15.7	15.8

Table 24. Mean predicted residues, uncorrected for recovery, with corresponding 95 %/95 % tolerance limits:

Day	Muscle µg/kg		Kidney µg/kg		Liver µg/kg		Renal fat µg/kg		Subcutaneous fat µg/kg	
	Predicted residue	95%/95%	Predicted residue	95%/95%	Predicted residue	95%/95%	Predicted residue	95%/95%	Predicted residue	95%/95%
0	364.47	1231.74	1315.61	2945.49	3038.27	5866.36	12284.1	24752.2	5653.93	13199.6
1	285.14	926.78	1016.84	2218.54	2345.23	4433.78	9658.84	19030.4	4936.99	11217.0
2	223.08	698.57	785.92	1672.99	1810.27	3354.27	7594.62	14646.3	4310.95	9544.01
3	174.52	527.78	607.44	1263.53	1397.34	2540.79	5971.55	11287.3	3764.30	8133.76
4	136.54	399.97	469.49	956.22	1078.60	1927.78	4695.36	8714.00	3286.97	6946.66
5	106.82	304.32	362.87	725.58	832.57	1465.84	3691.90	6742.87	2870.17	5949.39
6	83.57	232.75	280.46	552.46	642.66	1117.71	2902.89	5233.20	2506.22	5113.71
7	65.38	179.18	216.77	422.45	496.06	855.26	2282.51	4076.73	2188.42	4415.32
8	51.15	139.00	167.54	324.69	382.91	657.16	1794.71	3189.93	1910.91	3832.81
9	40.02	108.74	129.49	250.95	295.57	507.26	1411.16	2508.20	1668.60	3346.79
10	31.31	85.80	100.08	195.05	228.15	393.35	1109.57	1981.78	1457.01	2939.65
11	24.49	68.22	77.36	152.38	176.11	306.28	872.44	1572.80	1272.26	2595.93
12	19.16	54.59	59.79	119.55	135.94	239.33	685.99	1252.89	1110.93	2302.78
13	14.99	43.91	46.21	94.12	104.93	187.54	539.39	1001.03	970.06	2050.12
14	11.73	35.47	35.72	74.29	80.99	147.27	424.11	801.65	847.05	1830.27
15	9.18	28.73	27.60	58.76	62.52	115.84	333.47	643.11	739.64	1637.49
16	7.18	23.33	21.34	46.55	48.26	91.23	262.21	516.62	645.85	1467.39

The second study (Le, 2015) was conducted using 20, 9-month old crossbred beef cattle. Cattle were treated orally three times at a nominal dose of 3.75 mg monepantel/kg body weight and again doses were administered 21 days apart. Animals were slaughtered 21, 42, 56 and 85 days after the final treatment. Samples of muscle, kidney, liver, renal fat and subcutaneous fat were collected and analysed for monepantel sulphone residues.

The maximum monepantel sulphone residues were observed in the Day 21 samples: 1010, 583, 143, 63.4 and 14.5 µg/kg for subcutaneous fat, renal fat, liver, kidney and muscle, respectively. At the final sampling time, all residues were below the method limit of quantification (LOQ=5 µg/kg) (Table 25).

Table 25. Residues of monepantel sulphone in tissues of cattle treated three times, 21 days apart, with a nominal dose of 3.75 mg monepantel/kg body weight.

Day post third treatment	Animal no.	Liver	Kidney	Muscle	Subcutaneous fat	Renal fat
21	301	98.6	40.2	10.8	604	409
	302	78.1	32.7	7.01	340	398
	304	143	63.4	14.5	1010	583
	323	54.8	27.5	6.90	375	348
	324	68.0	28.3	10.7	457	344
42	305	6.58	<LOQ (4.02, 3.94*)	<LOQ (1.58)	29.7	17.4
	307	6.68	<LOQ (3.71, 3.62*)	<LOQ (1.76)	28.0	22.7
	308	6.14	<LOQ (3.62, 3.06*)	<LOQ (1.75)	22.9	29.1
	313	<LOQ (4.37)	<LOQ (2.94, 2.83*)	<LOQ (1.50)	15.9	16.2
	316	<LOQ (1.84)	<LOQ (1.64, 1.47*)	<LOQ (1.21)	9.08	6.28
56	310	5.01	<LOQ (2.94)	<LOQ (2.20)	23.8	18.0
	312	<LOQ (2.60)	<LOQ (1.86)	<LOQ (1.32)	8.11	7.83
	315	<LOQ (1.08)	<LOQ (1.64)	<LOQ (1.01)	<LOQ (4.36)	<LOQ (4.53)
	319	<LOQ (0.705)	<LOQ (1.09)	<LOQ (1.04)	<LOQ (2.01)	<LOQ (2.14)
	322	<LOQ (2.81)	<LOQ (1.17)	<LOQ (1.10)	<LOQ (4.07)	<LOQ (3.49)
85	306	<LOQ (0.555)	Not analyzed	Not analyzed	<LOQ (0.669)	<LOQ (0.696)
	309	<LOQ (0.492)			<LOQ (0.901)	<LOQ (0.599)
	314	<LOQ (0.623)			<LOQ (0.778)	<LOQ (1.28)
	317	<LOQ (0.531)			<LOQ (0.715)	<LOQ (0.553)
	321	<LOQ (0.929)			<LOQ (0.950)	<LOQ (0.556)

*reanalyzed for confirmation

The incurred residues at the first sampling time in the second study (21 days post-last dose, Le, 2015) were generally consistent with the incurred residues at the last sampling time in the first study (13 days post-last dose, Adams and Le, 2014) and continued the depletion trend (Table 26).

Table 26. Comparison of the incurred residues at the final sampling time of the first study (Adams and Le, 2014) with those at the first sampling time of the second study (Le, 2015).

	Kidney	Liver	Perirenal fat	Subcutaneous Fat	Muscle
Day 13 (Adams and Le, 2014)	43.00	102.80	553.00	1157.20	14.10
Day 21 (Le, 2015)	38.42	88.50	416.40	557.20	9.98

Lactating dairy cows

No unlabelled residue data were provided for the use of monepantel in lactating dairy cattle.

Sheep

The depletion of unlabelled monepantel in tissues of sheep was evaluated at the 75th meeting of the Committee (FAO, 2011)

Methods of analysis for residues in tissues

An analytical procedure, validated under GLP conditions (Browning, 2014a), with a LOQ of 5 µg/kg and demonstrated to be suitable for routine analysis of monepantel in bovine tissue matrices, was available that met the requirements of the validation criteria established by the CCRVDF, as contained in CAC/GL 71-2009.

Briefly, the validated method for the extraction and analysis of monepantel residues in bovine tissue matrices is as follows: The ground tissue sample (0.5 g) is extracted with ACN (5 mL), either by mechanical homogenization for 2 min (for non-fat tissues) or by mechanical shaking for 10 min followed by ultra-sonication for 10 min (for fat). The mixture is centrifuged briefly, and the supernatant is diluted to 50 mL with ACN/MeOH/water. For samples with residues above 750 µg/kg, a further dilution is conducted (1:40 in the same solvent). Once diluted, the extract is injected onto the LC-MS/MS, which is calibrated with injections of varying concentrations of pure standards (0.05 to 7.5 ng/mL, equivalent to 5 to 750 µg/kg, for extracts not requiring further dilution).

Reversed-phase HPLC was conducted using a Waters Atlantis® T3 (3 µm, 2.1 X 50 mm; part number 186003717) column with a Waters Atlantis® T3 (3 µm, 2.1x10 mm; part number 186003756) guard column. The mobile phase A: 20 mM ammonium bicarbonate in water with 5 % acetonitrile, mobile phase B: acetonitrile/methanol (50/50, v/v), flow rate 0.3 mL with gradient elution were used to chromatographically separate monepantel sulphone from any other extracted components with detection by tandem MS with electrospray ionization in the negative mode. Monepantel sulphone was quantified with the transition ion (m/z 503.8 → 186) and its identity confirmed with the two qualifying transitions at m/z 504 → 166 and m/z 504 → 146 at the appropriate chromatographic retention time. Quantification in unknown samples was done by comparing the analyte area responses to those of the calibration curve generated by linear regression with 1/x weighting (origin excluded).

Selectivity

Control tissues from 20 different sources for each tissue type were analysed for interference by co-extractives and compared to the lowest STD. Mean interferences at the retention time were less than 20 % of the lowest standard at 5 µg/kg.

Solutions of fenbendazole, thiabendazole, triclabendazole, triclabendazole sulphone, triclabendazole sulfoxide, levamisole, fluazuron, ivermectin, abamectin, doramectin, moxidectin, amoxicillin, oxytetracycline, ceftiofur, florfenicol, cypermethrin and monepantel each at a concentration equivalent to approximately 1000 µg/kg were injected and analysed for a response at the retention time and transition of the marker residue. Minor interference of 22 % of the lowest standard at 5 µg/kg was observed for levamisole when it was injected as a single, neat solution at a 200-fold higher concentration of the target analyte.

Matrix samples containing the selected veterinary drugs including monepantel each at a concentration of 1000 µg/kg were extracted and fortified thereafter with monepantel sulphone at 350 µg/kg. The responses from these samples were compared to matrix-matched and matrix-fortified extracts at the same concentrations. The detector response for monepantel sulphone did not show any suppression or enhancement in presence of the other drugs.

These experiments confirmed that the method was selective and that the method will accurately detect negative control samples as negative and that the presence of other drugs such as fenbendazole, thiabendazole, triclabendazole, triclabendazole sulphone, triclabendazole sulfoxide, levamisole, fluazuron, ivermectin, abamectin, doramectin, moxidectin, amoxicillin, oxytetracycline, ceftiofur, florfenicol, cypermethrin or their metabolites and the parent compound, monepantel, used in food animal production will not interfere with the quantification of monepantel sulphone. Other metabolites, as seen in the ADME cattle study (Vance, 2014), are well separated from the analyte on reversed-phase HPLC and should not interfere. The method, however, is not stereo-specific, so the other enantiomer of monepantel sulphone also would be detected but, because it was determined in the sheep ADME study (FAO, 2011) that this isomer is not formed, it is likely that this will also be true for cattle.

Recovery

The total recovery was obtained by comparison of the analytical response of blank matrix spiked with the analyte and extracted compared to the response of a STD solution analysed directly.

The extraction loss was obtained by comparison of the analytical response of a blank matrix sample spiked with the analyte and extracted compared to the response of blank matrix of the same origin first extracted and then spiked post-extraction with the analyte.

The matrix effect was obtained by comparison of the analytical response of blank matrix first extracted and then spiked 'post extraction' with the analyte compared to the response of a STD solution analysed directly (Table 27).

Table 27. Matrix effects and recovery of extraction from bovine tissue matrices taken from six different matrix sources for each tissue type and fortified at the LOQ of the method at a concentration of 5 µg/kg

Matrix	Fat	Liver	Kidney	Muscle
Qualitative Matrix Effect	Suppression	Suppression	Suppression	Enhancement
Mean Matrix Effect (%)	-7.42	-3.56	-0.391	-5.27
Mean Extraction loss (%)	-1.64	-7.49	-3.28	-12.0
Mean Total Recovery (%)	91.1	89.2	96.3	92.6

Accuracy

The accuracy data presented in Tables 28-31 for fat, liver, kidney and muscle tissues respectively, demonstrated that the method was accurate and met the acceptance criteria for accuracy: 60–120 % for concentrations ≥ 1 µg/kg < 10 µg/kg; 70–110 % for concentrations ≥ 10 µg/kg < 100 µg/kg; and 80–110 % for concentrations ≥ 100 µg/kg.

The precision data shown in Tables 28-31 for fat, liver, kidney and muscle tissues, respectively, showed that the method met the acceptance criteria for intra-day variability (CV): intra-day CV were all ≤ 25 % for all concentrations ≥ 1 µg/kg < 10 µg/kg, ≤ 15 % for concentrations ≥ 10 µg/kg < 100 µg/kg, and ≤ 10 % for concentrations ≥ 100 µg/kg.

The inter-day variability CV were all ≤ 32 % for concentrations ≥ 1 µg/kg < 10 µg/kg, ≤ 23 % for concentrations ≥ 10 µg/kg < 100 µg/kg, and ≤ 16 % for concentrations ≥ 100 µg/kg.

Table 28. Accuracy & Precision Data for Fat

Fat batch	Result (µg/kg)	Accuracy (%)	Result (µg/kg)	Accuracy (%)	Result (µg/kg)	Accuracy (%)
QC (µg/kg)		5.00		350.0		750.0
Intra-day 1 mean	5.36	106	328	91.7	709	94.9
CV (%)	0.56	10.3	15.8	4.82	23.3	3.31
Intra-day 2 mean	4.72	93.4	348	97.2	744	99.5
CV (%)	0.27	5.70	19.4	5.45	35.3	4.69
Intra-day 3 mean	5.04	99.9	335	93.7	718	96.1
CV (%)	0.59	11.7	12.8	3.82	244	3.45
Inter-day mean	5.04	100	337	94.2	724	96.8
CV (%)	0.53	10.6	17.4	5.10	30.6	4.22

Table 29. Accuracy & Precision data for Liver

Liver batch	Result (µg/kg)	Accuracy (%)	Result (µg/kg)	Accuracy (%)	Result (µg/kg)	Accuracy (%)
QC (µg/kg)		5.00		350.0		750.0
Intra-day mean	5.25	104	292	95.7	707	94.7
CV (%)	0.26	5.01	12.4	4.51	24	3.47
Intra-day mean	4.53	89.8	356	99.7	752	101
CV (%)	0.23	4.96	7.2	2.11	26	3.36
Intra-day mean	4.83	95.5	339	94.8	689	92.3
CV (%)	0.58	12.0	8.2	2.33	17.7	2.57
Inter-day mean	4.87	96.4	346	96.7	716	95.9
CV (%)	0.48	9.78	12.9	3.74	34.5	4.83

Table 30. Accuracy & Precision Data for Kidney

Kidney batch	Result (µg/kg)	Accuracy (%)	Result (µg/kg)	Accuracy (%)	Result (µg/kg)	Accuracy (%)
QC (µg/kg)		5.00		350.0		750.0
Intra-day mean	5.33	106	356	100	745	100
CV (%)	0.31	5.93	10.4	3.00	21.9	2.95
Intra-day mean	4.80	95.0	351	98.3	720	96.4
CV (%)	0.17	3.44	6.9	2.04	8.2	1.15
Intra-day mean	5.36	106	331	92.5	701	93.8
CV (%)	0.14	2.70	8.0	2.37	16.6	2.37
Inter-day mean	4.89	102	347	96.9	722	96.7
CV (%)	1.04	6.59	14.2	4.17	24.4	3.40

Table 31. Accuracy & Precision Data for Muscle

Muscle batch	Result (µg/kg)	Accuracy (%)	Result (µg/kg)	Accuracy (%)	Result (µg/kg)	Accuracy (%)
QC (µg/kg)	5.00		350.0		750.0	
Intra-day mean	4.65	92.0	342	95.5	731	97.9
CV (%)	0.44	9.41	3.8	1.07	15.8	2.20
Intra-day mean	4.71	93.3	348	97.4	699	93.6
CV (%)	0.24	5.23	16	4.52	15.4	2.19
Intra-day mean	4.49	88.9	295	82.4	644	86.2
CV (%)	0.399	8.91	17.6	6.02	16.7	2.58
Inter-day mean	4.62	91.4	328	91.8	691	92.6
CV (%)	0.36	7.82	27.7	8.44	40	5.80

Stability

Monepantel sulphone was demonstrated to be stable when stored in the auto sampler chamber at room temperature for 3 days and at approximately 15 °C for 4 days and in the cool room at approximately 6 °C for 6 days.

To evaluate the stability in matrix extract, triplicate incurred samples of fat, liver, kidney and muscle were homogenized with extraction solvent and analysed following storage of extracts in capped polypropylene centrifuge tubes stored at approximately 6 °C for 2 weeks.

To evaluate stability, five subsamples of incurred residue samples for each tissue was analysed within 1 week of receipt; other samples were stored for the evaluation of short term bench top (4 h at room temperature prior to addition of solvent), over 3 freeze/thaw cycles and long-term storage stability (up to 7.5 months continually frozen at approximately -10 to -22 °C).

Monepantel sulphone was demonstrated to be stable when stored under all the above-specified conditions using incurred material in the studies (Table 32).

Table 32. Results of Stability Studies

Batch	Matrix Time point	Fat Residue (µg/kg)	Liver Residue (µg/kg)	Kidney Residue (µg/kg)	Muscle Residue (µg/kg)
Initial		608	222	80.7	22.7
Concentration (1 week)	Mean				
	CV (%)	2.67	1.22	2.14	5.78
Reference value	T ₀ Accuracy (%)	100	100	100	100
Extracts 14 days at ~6 °C	Mean	608	212	67.5	19.0
	CV (%)	1.24	0.817	2.96	8.97
	**Accuracy (%)	106	99.5	98.8	91.8
	Mean	594	208	70.3	22.0
	CV (%)	1.22	2.17	3.56	6.46

4 h ambient prior to extraction	*Accuracy (%)	97.7	93.7	87.2	96.8
Freeze/thaw 3 cycles	Mean	595	214	65.7	21.1
	CV (%)	1.85	0.540	2.04	1.42
	*Accuracy (%)	97.9	96.4	81.5	93.0
	**Accuracy (%)	104	100	96.2	102
Continually frozen 3 months	Mean	575	213	68.3	20.7
	CV (%)	3.95	3.39	2.93	3.70
	*Accuracy (%)	94.5	96.1	84.7	91.0
Continually frozen 4 months	Mean	589	212	66.7	21.0
	CV (%)	1.79	1.25	6.32	5.24
	*Accuracy (%)	96.8	95.7	82.7	92.5
Continually frozen 7.5 months	Mean	578	197	65.5	21.6
	CV%	2.77	2.33	14.6	4.99
	*Accuracy (%)	95.1	88.9	81.2	95.3

* Accuracy compared to initial analysis (T₀) ** Accuracy compared to the 3 month frozen cycle results

Appraisal

Monepantel was previously evaluated by the Committee for use in sheep. Monepantel for use in cattle was included in the agenda for the current meeting of the Committee at the request of the 23rd Session of the CCRVDF. Monepantel is an anthelmintic of the amino-acetonitrile derivative class indicated for the treatment of roundworms in cattle. The recommended dose is 2.5 mg/kg bw and the maximum dose used is 3.7 mg/kg bw. Up to three doses per season can be applied with a minimum retreatment interval of 21 days.

Two GLP-compliant ADME studies were evaluated. In the first study, cattle were treated orally with [¹⁴C]-monepantel (3.75 mg/kg bw). Systemic absorption was relatively high, with TRR reaching peak concentrations at 24 h after dosing. Initial elimination was rapid with a half-life of about 36 h, but this slowed progressively at later times. Residues were still detectable at the final sampling time point at *ca.* 5 µg equivalents/kg in blood and plasma. About 21 % of the dose was eliminated in the urine over 3 days. Approximately 36 % of the dose was eliminated in the faeces. About 60 % of the dose was recovered in excreta over 3 days, with the remaining material distributed in the tissues.

In the second GLP compliant study, conducted as part of the pivotal cold residue depletion study, the pharmacokinetic profiles of monepantel and monepantel sulphone in blood were investigated in cattle orally dosed three times 21 days apart at 3.75 mg/kg bw monepantel. The blood samples were analysed for monepantel and monepantel sulphone using a validated LC-MS/MS method. Monepantel sulphone was the dominant residue in the blood. The blood profiles of the animals were compared and no accumulation of monepantel and monepantel sulphone in blood was evident. Additionally, the blood profile (measuring individual

metabolites) was similar to that observed in the cattle ADME study where only TRRs were measured.

Edible tissues, blood, bile and excreta from the GLP-compliant radiolabelled ADME study in cattle were investigated for extractability of residues, and metabolite profiles. Radioactivity was extracted readily from fat, kidney and muscle, with simple solvent extraction. Liver residues proved more difficult to extract, especially with increasing time after dose administration.

The major component present in all tissues cochromatographed with monepantel sulphone. The parent molecule, monepantel, was a minor constituent. Minor unknown components of high polarity were detected in the liver, kidney and muscle.

A non-GLP in vitro metabolism study was conducted to investigate the metabolism of monepantel in intact rat, cattle and sheep hepatocytes. Incubations were profiled by HPLC and profiles were similar across the tested species. The results of the in vitro study were consistent with those in the in vivo ADME study.

Selected samples of edible tissues from the radiolabelled GLP-compliant ADME study in cattle were investigated for TRR. The calculated half-lives of TRR were 4-5 days for fat, kidney and muscle but *ca.* 10 days for liver, consistent with the presence of bound residues. The order of residues in tissues is fat > liver > kidney > muscle, indicative of lipophilic substances, and is the same as that seen for sheep. Because the major residue is monepantel sulphone, it is the marker residue identified for monepantel in cattle tissues. It also is the marker identified previously for sheep tissues. Concentrations of the monepantel sulphone residues in cattle tissue were determined as part of the method validation study and used to determine the MR to TRR ratio cattle tissues.

Two GLP-compliant residue depletion studies were conducted in cattle using unlabelled monepantel.

The first study was conducted using crossbred beef cattle. Cattle were treated orally three times at a nominal dose of 3.75 mg monepantel/kg body weight. Doses were administered 21 days apart. Animals were slaughtered 4, 7, 10 and 13 days after the final treatment. Samples of muscle, kidney, liver, renal fat and subcutaneous fat were collected and analysed for monepantel sulphone.

The maximum monepantel sulphone residues were observed in the Day 4 samples. While initial residues in renal fat were highest, by the later sampling times residues were highest in subcutaneous fat. The calculated half-life estimates for muscle, kidney, liver, renal and subcutaneous fat were 2.8, 2.7, 2.7, 2.9 and 5.1 days, respectively.

The second study also was conducted using crossbred beef cattle. Again, cattle were treated orally three times at a nominal dose of 3.75 mg monepantel/kg body weight and again doses were administered 21 days apart. In this second study, animals were slaughtered 21, 42, 56 and 85 days after the final treatment. Samples of muscle, kidney, liver, renal fat and subcutaneous fat were collected and analysed for monepantel sulphone residues.

The maximum monepantel sulphone residues were observed in the Day 21 samples. At the final sampling time, all residues were below the method limit of quantification (LOQ=5 µg/kg).

Using the data from the first GLP-compliant residue depletion study, the upper one-sided 95 % confidence interval over the 95th percentile of the residue concentrations was calculated for each edible tissue. The ratio of the mean concentration of the marker residue to that of the TRR was calculated as 0.88 in fat, 0.2 in liver and 0.62 in kidney. The ratio of the concentration of marker residue to total residues in muscle is available at only the first sampling time and is 0.67. Additionally, a correction factor of 0.94 to account for the mass difference between monepantel sulphone (the marker residue) and monepantel was applied.

A validated LC-MS/MS, demonstrated to be suitable for routine analysis of monepantel in bovine tissue matrices, was available that met the requirements of the validation criteria established by the CCRVDF, as contained in CAC/GL 71-2009.

Maximum Residue Limits

In recommending MRLs for monepantel in cattle, the Committee considered the following factors:

- An ADI of 0-0.02 mg/kg bw was previously established by the Committee.
- An Acute Reference Dose was considered unnecessary.
- The metabolite, monepantel sulphone, is the marker residue in cattle tissues.
- Fat contains the highest concentration of monepantel sulphone at all sampling times, followed by liver, kidney and muscle. Liver and fat can serve as the target tissues.
- The ratios of the concentration of marker residue to total residues are 0.88 in fat, 0.20 in liver and 0.62 in kidney at 7 days post-treatment. The ratio of the concentration of marker residue to total residues in muscle is available only at the first sampling time, 3 days post-treatment, and is 0.67.
- A correction factor of 0.94 is applied to account for the mass difference between monepantel sulphone (the marker residue) and monepantel.
- A validated analytical method for the determination of monepantel sulphone in edible cattle tissues (liver, kidney, muscle and fat) is available and may be used for monitoring purposes.
- MRLs were calculated on the basis of the upper limit of the one-sided 95 % confidence interval over the 95th percentile of the residue concentrations (UTL 95/95)

Consistent with the conditions of good veterinary practice in the Member State with an approved use of monepantel in cattle, the Committee recommended MRLs determined as monepantel sulphone, expressed as monepantel, in cattle tissue of 300 µg/kg in muscle, 1000 µg/kg in kidney, 2000 µg/kg in liver and 7000 µg/kg in fat.

Exposure Assessment

Exposure to monepantel residues is considered to occur only through its use as a veterinary drug in the muscle, liver, kidney and fat of sheep and other ovines and cattle and other bovines.

In the JECFA dietary exposure assessment, sheep and other ovine muscle, liver, kidney and fat and cattle and other bovine muscle, liver, kidney and fat, were contributors to dietary exposure. Where appropriate consumption data for a specific category (e.g., ovine kidney) were not available, the more generic category (e.g., mammalian kidney) was used in conjunction with the associated highest residue to estimate exposure.

The GECDE for monepantel was calculated based on median residues (STMR) 7 days or 5 days after administration of the drug in sheep and cattle respectively. The STMR in the specific tissues of both species were included in the exposure estimate wherever possible. In all other cases, the highest STMR from either species was used together with the most appropriate food consumption available for the tissue of interest.

The GECDE for the general population is 13.7 µg per kg bodyweight per day, which represents 68 % of the upper bound of the ADI of 20 µg per kg bw. The GECDE for children is 5.0 µg per kg bodyweight per day, which represents 25 % of the upper bound of the ADI of 20 µg per kg bw. The GECDE for infants is 4.4 µg per kg bodyweight per day, which represents 22 % of the upper bound of the ADI of 20 µg per kg.

Table 33. Exposure Assessment

Category	Type	Median concentration ¹ (µg/kg)	Mean consumption ² (whole population, g/kg bw)	Highest reliable percentile consumption ³ (consumers only, g/kg bw) / [percentile used]	MR:TR ratio	Correction	Exposure (µg/kg bw/day)		(µg/kg)		GECD ⁴	
							mean	Highest Reliable Percentile	µg/k g bw/d	% ADI		
General Population												
Mammalian muscle	Sheep and other ovines	152	0.163	4.320 [97.5]	1.00	0.94	0.023	0.617				
Mammalian offal	Cattle liver	831	0.028	3.322 [97.5]	0.2	0.94	0.110	12.976				
Mammalian offal	Cattle Kidney	362	0.039	3.976 [97.5]	0.62	0.94	0.021	2.182				
Mammalian fat ⁶	Sheep fat	2660	0.002	0.105 [97.5]	0.66	0.94	0.006	0.399				
Mammalian muscle	Beef and other Bovines	107	0.959	4.442 [97.5]	0.67	0.94	0.144	0.667				
Mammalian offal	Sheep liver	1259	0.009	2.103 [97.5]	0.66	0.94	0.017	3.879				
Mammalian offal	Sheep Kidney	406	0.160	0.105 [97.5]	0.66	0.94	0.001	0.062				
Mammalian fat ⁶	Cattle fat	3257	0.143	0.610 [97.5]	0.88	0.94	0.497	2.124				
TOTAL							0.709	12.976			13.68	68.4
Children												
Mammalian muscle	Sheep and other ovines	152	0.353	9.425 [97.5]	1.00	0.94	0.050	1.347				
Mammalian offal	Cattle offal ⁷	831	0.049	0.504 [97.5]	0.20	0.94	0.193	1.969				
Mammalian fat ⁶	Sheep fat	2660	0.001	0.146 [97.5]	0.66	0.94	0.005	0.554				
Mammalian muscle	Beef and other Bovines	107	1.940	8.394 [97.5]	0.67	0.94	0.291	1.260				

Table 33. Exposure Assessment

Category	Type	Median concentration ¹ (µg/kg)	Mean consumption ² (whole population, g/kg bw)	Highest reliable percentile (consumers only, g/kg bw) [percentile used]	MR:TR ratio	Correction	Exposure (µg/kg bw/day)		GECDE ⁴	
							mean	Highest Reliable Percentile	µg/k g bw/d ay	% ADI
Mammalian offal	Sheep offal ⁷	1259	0.001	0.001 [97.5]	0.66	0.94	0.013	0.002		
Mammalian fat ⁶	Cattle fat	3257	0.220	1.268 [97.5]	0.88	0.94	0.765	4.411		
TOTAL							0.552	4.411	4.964	24.8
Infants⁸										
Mammalian muscle	Sheep and other ovines ⁹	152	0.115	4.571 [97.5]	1.00	0.94	0.016	0.653		
Mammalian muscle	Beef and other Bovines	107	0.278	5.842 [97.5]	0.67	0.94	0.042	0.876		
Mammalian offal	Mammalian offal	1295	0.003	1.110 [97.5]	0.20	0.94	0.010	4.336		
TOTAL							0.058	4.336	4.394	22.0

¹Median concentration at the end of treatment ²highest mean consumption figures based on whole population considered from the available dataset ³highest food consumption figures based on consumers only considered from the available dataset ⁴GECDE is the sum of the highest exposure at the highest reliable percentile of consumption for a food and the mean dietary exposures of the other foods ⁵Mammalian trimmed fat, skin and added fat ⁶Mammalian trimmed fat, skin and added fat excluding butter ⁷No highest reliable percentiles for kidney and liver available, highest residue was used for offal consumption⁸No consumption of mammalian fat was reported for infants ⁹based on consumption of unprocessed meat and offal ¹⁰No highest reliable percentiles for kidney and liver available, no offal consumption at species level available, highest residue was used with mammalian offal consumption

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Annex 1 – Summary of recommendations from the 85th JECFA on compounds on the agenda and further information required

Amoxicillin (antimicrobial agent)

Acceptable daily intake	The Committee established a microbiological ADI (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 global estimate of chronic dietary exposure (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	<p>The global estimate of acute dietary exposure (GEADE) for the general population is 1.4 µg/kg bw, which represents 28 % of the microbiological ARfD.</p> <p>The GEADE for children is 1.6 µg/kg bw, which represents 31 % of the microbiological ARfD.</p>
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 no-observed-adverse-effect level (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.

Future work and recommendations

Information essential in the evaluation of the compound

In order to determine suitable marker residue(s), a metabolism study using radiolabelled ethion in cattle is required

- to determine the ratios of the parent compound and metabolites (i.e. potential marker residues) to the total residues over the residue depletion period in edible tissues (e.g. liver, kidney, muscle, fat), and
- to identify the metabolites.

Cattle metabolites should be compared with laboratory species metabolites to ensure that all residues of toxicological concern produced in cattle have been covered by the available toxicology studies.

Analytical method(s) that can measure suitable marker residues in all edible tissues should be developed and validated in accordance with established guidance (CAC/GL71-2009).

As the ADI for ethion was based on developmental effects and is 10-fold lower than the ARfD, specific exposure scenarios are required to address exposure in high-percentile pregnant consumers or a suitable surrogate population. This exposure scenario will also be protective of children.

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 two-generation 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 displayed in the JECFA 85 report. Results below are only for use as 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.

Halquinol (broad spectrum antimicrobial)

Acceptable daily intake

In the absence of information required to assess the in vivo mutagenicity and carcinogenicity potential of halquinol, the Committee was unable to establish an ADI for halquinol.

An mADI of 0–0.3 mg/kg bw was derived from in vitro MIC susceptibility testing data.

Acute reference dose

A microbiological ARfD of 0.9 mg/kg bw was established based on the effects of halquinol on the intestinal microbiota.

Estimated dietary exposure

No dietary exposure assessment could be conducted.

Residue definition

None due to incomplete characterization of residues in tissues.

Maximum residue limits The Committee was unable to recommend MRLs for halquinol.

Future work and recommendations

Further information required to complete the residue assessment:

- Characterization of the non-extractable radiolabelled residues in tissues as well as the extractable (but not defined) residues;
- An accurate marker residue to total radioactive residue (MR:TRR) ratio over the appropriate time in pig edible tissues; and
- Further characterization of halquinol metabolites in tissues.

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 displayed in the JECFA 85 report. Results below are only for use as 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)

Species	Fillet^a (µg/kg)
Salmon	1 350
Trout	1 350

^a Muscle plus skin in natural proportion.

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 chronic dietary exposure	<p>The GECDE for the general population is 13.7 µg per kg bw per day, which represents 68 % of the upper bound of the ADI.</p> <p>The GECDE for children is 5.0 µg per kg bw per day, which represents 25 % of the upper bound of the ADI.</p> <p>The GECDE for infants is 4.4 µg per kg bw per day, which represents 22 % of the upper bound of the ADI.</p>
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.

Future work and recommendations***Further information that would assist in the further evaluation of the compound***

Comparative toxicokinetic data in rat, dog and human;

Effects of sisapronil at steady state following repeated-dose oral administration in the dog; and

Determination of the relevance of the effects on the thyroid observed in dogs.

Although not all the toxicokinetic data on sisapronil would have to be generated in vivo, the approach used would have to be suitably validated (e.g. physiologically based toxicokinetic model verified in vivo in rat and dog).

Zilpaterol hydrochloride (β_2 -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.

Annex 2 - Summary of JECFA evaluations of veterinary drug residues from the 32nd meeting to the present

The following table summarises the veterinary drug evaluations conducted by JECFA at the 32nd (1987), 34th (1989), 36th (1990), 38th (1991), 40th (1992), 42nd (1994), 43rd (1994), 45th (1995), 48th (1997), 50th (1998), 52nd (1999), 54th (2000), 58th (2002), 60th (2003), 62nd (2004), 66th (2006), 70th (2008), 75th (2011), 78th (2013), 81st (2015) and 85th (2017) meetings. These meetings were devoted exclusively to the evaluation of veterinary drug residues in food. This table must be considered in context with the full reports of these meetings, published as WHO Technical Report Series.

Some notes regarding the table:

- The “ADI/ArfD” column provides the ADI and, when applicable, the ArfD established by the Committee. When no ARfD is stated, an ArfD has not been established.
- The “ADI Status” column refers to the ADI and indicates whether an ADI was established; if a full ADI was given, or if the ADI is temporary (T).
- Where an MRL is temporary, it is indicated by “T”.
- Where a compound has been evaluated more than once, the data given are for the most recent evaluation, including the 78th meeting of the Committee.

Substance	ADI/ARfD (µg/kg bw)	ADI Status	JECFA	MRL (µg/kg)	Tissue	Species	Marker residue and other remarks
Abamectin	ADI: 0-1 (JMPPR 1995)	Full	47 (1996)	100	Liver, Fat	Cattle	Avermectin B _{1a}
Albendazole	ADI: 0-50	Full	34 (1989)	100	Muscle, Fat, Milk	Cattle, Sheep	MRLs analysed as 2- amino-benzimidazole, expressed as albendazole equivalents
Amoxicillin	ADI: 0-0.7	Full	75 (2011)	50	Muscle, Liver, Kidney, Fat	Cattle, Pig, Sheep	Amoxicillin
				4	Milk	Cattle, Sheep	
	ADI: 0-2	Full	85 (2017)	50	Muscle ^b	Finfish ^a	Amoxicillin
Ampicillin	ADI: 0-3	Full	85 (2017)	50	Muscle ^b	Finfish ^a	Ampicillin
Apramycin	ADI: 0-30	Full	75 (2011)	5000	Kidney	Cattle, Chicken	Apramycin
Avilamycin	ADI: 0-2000 (as avilamycin activity)	Full	70 (2008)	200	Muscle, Kidney, Skin/Fat	Pig, Chicken, Turkey, Rabbit	Dichloroisovevermic acid (DIA), expressed as avilamycin equivalents
				300	Liver	Pig, Chicken, Turkey, Rabbit	
Azaperone	ADI: 0-6	Full	52 (1999)	60	Muscle, Fat	Pig	Sum of azaperone and azaperol
				100	Liver, Kidney		
Benzylpenicillin	ADI: <30µg/person/ day of the penicillin moiety	Full	36 (1990)	50	Muscle, Liver, Kidney		Benzylpenicillin
				4	Milk	All species	

Substance	ADI/ARfD (µg/kg bw)	ADI Status	JECFA	MRL (µg/kg)	Tissue	Species	Marker residue and other remarks
Bovine Somatotropins	ADI: Not specified	Full	78 (2013)	Not specified	Muscle, Liver, Kidney, Fat, Milk	Cattle	
Carazolol	ADI: 0-0.1 ARfD: 0.1	Full	52 (1999)	5	Muscle, Fat/Skin		Carazolol. The Committee noted that the concentration of carazolol at the injection site may exceed the ADI that is based on the acute pharmacological effect of carazolol.
Carbadox	No ADI or ARfD		60 (2003)	No MRL	Liver, Kidney	Pig	The Committee decided that quinoxaline-2- carboxylic acid is not an appropriate marker residue
Ceftiofur	ADI: 0-50	Full	48 (1997)	1000 2000 6000 100	Muscle Liver, Fat Kidney Milk	Cattle, Pig	Desfuroylceftiofur
Cefuroxime	No ADI or ARfD		62 (2004)	No MRL			
Chloramphenicol	No ADI or ARfD		62 (2004)	No MRL			
Chlorpromazine	No ADI or ARfD		38 (1991)	No MRL			
Chlortetracycline, Oxytetracycline, Tetracycline	ADI: 0-30 (Group ADI)	Full	58 (2002)	200 600 1200	Muscle Liver Kidney	Cattle, Pig, Sheep, Poultry	Parent drugs, either singly or in combination

Substance	ADI/ARfD (µg/kg bw)	ADI Status	JECFA	MRL (µg/kg)	Tissue	Species	Marker residue and other remarks
Closantel	ADI: 0-30	Full	40 (1992)	1000	Muscle, Liver	Cattle	Closantel
				3000	Kidney, Fat		
				1500	Muscle, Liver	Sheep	
				5000	Kidney		
				2000	Fat		
Colistin	ADI: 0-7	Full	66 (2006)	150	Muscle, Liver, Fat	Cattle, Sheep, Goat, Chicken, Turkey,	Residue definition is the sum of Colistin A and colistin B. The MRL includes skin + fat where appropriate (chicken, turkey, pigs).
				200	Kidney	Pig, Rabbit	
				50	Milk	Cattle, Sheep	
				300	Eggs	Chicken	
				20	Muscle,	Liver,	
Cyfluthrin	ADI: 0-20	Full	48 (1997)	200	Kidney		Cyfluthrin
				40	Fat	Cattle	
					Milk		
Clenbuterol	ADI: 0-0.004	Full	47 (1996)	0.2	Muscle, Fat	Cattle, Horse	Oxytetracycline only Clenbuterol
				0.6	Liver, Kidney	Cattle, Horse	
				0.05	Milk	Cattle	
Eggs				400	Eggs	Poultry	
				100	Milk	Cattle, Sheep	
				200	Muscle	Fish, Giant prawn	

Substance	ADI/ARfD (µg/kg bw)	ADI Status	JECFA	MRL (µg/kg)	Tissue	Species	Marker residue and other remarks
Cyhalothrin	ADI: 0-5	Full	62 (2004)	20	Muscle, Kidney	Cattle, Sheep, Pig	Cyhalothrin
				400	Fat	Cattle, Sheep, Pig	
				20	Liver	Cattle, Pig	
				50	Liver	Sheep	
				30	Milk	Cattle, Sheep	
Cypermethrin	ADI: 0-20	Full	62 (2004)	50	Muscle,	Liver, Cattle, Sheep	Total of cypermethrin residues (resulting from the use of cypermethrin or α -cypermethrin as veterinary drugs)
α -Cypermethrin	(Group ADI)			1000	Kidney		
				100	Fat		
					Milk		
Danofloxacin	ADI: 0-20	Full	48 (1997)	200	Muscle	Cattle, Chicken	Danofloxacin For chicken fat/skin in normal proportions
				400	Liver, Kidney		
				100	Fat		
				100	Muscle		
				50	Liver		
				200	Kidney		
				100	Fat		
Deltamethrin	ADI: 0-10 (1982 JMPR)	Full	60 (2003)	30	Muscle	Cattle, Chicken, Sheep, Salmon	Deltamethrin
				50	Liver, Kidney	Cattle, Sheep,	
				500	Fat	Chicken	

Substance	ADI/ARfD (µg/kg bw)	ADI Status	JECFA	MRL (µg/kg)	Tissue	Species	Marker residue and other remarks
Derquantel	ADI: 0-0.3	Full	78 (2013)	30	Milk	Cattle	Derquantel
				30	Eggs	Chicken	
				0.3	Muscle	Sheep	
				0.4	Kidney		
Dexamethasone	ADI: 0-0.015	Full	70 (2008)	7.0	Fat		Dexamethasone
				0.8	Liver		
				1	Muscle, Kidney	Cattle, Pig, Horse	
				2	Liver	Cattle, Pig, Horse	
Diclazuril	ADI: 0-30	Full	50 (1998)	0.3	Milk	Cattle	Diclazuril Poultry skin + fat
				500	Muscle	Sheep, Rabbit,	
				3000	Liver	Poultry	
				2000	Kidney		
Diflubenzuron	No ADI or ARfD	Full	81 (2015)	1000	Fat		Sum of dihydrostreptomycin and streptomycin
				600	Muscle, Liver, Fat	Cattle, Pig, Chicken,	
				1000	Kidney	Sheep	
				200	Milk	Cattle, Sheep	
Dimetridazole	No ADI or ARfD		34 (1989)	No MRL			
Diminazene	ADI: 0-100	Full	42 (1994)	500	Muscle		Diminazene
				12000	Liver	Cattle	
				6000	Kidney		

Substance	ADI/ARfD (µg/kg bw)	ADI Status	JECFA	MRL (µg/kg)	Tissue	Species	Marker residue and other remarks
Doramectin	ADI: 0-1	Full	62 (2004)	150	Milk		
				10	Muscle	Cattle	Doramectin
				5	Muscle	Pigs	
				100	Liver	Cattle, Pigs	
				30	Kidney	Cattle, Pigs	
				150	Fat	Cattle, Pigs	
				15	Milk	Cattle	
Enamectin benzoate	ADI: 0-0.5	Full	78 (2013)	100	Muscle	Salmon	Enamectin B _{1a}
					Fillet (muscle with skin)	Trout	
Enrofloxacin	ADI: 0-2	Full	48 (1997)	No MRL			
Eprinomectin	ADI: 0-10	Full	50 (1998)	100	Muscle	Cattle	Eprinomectin B _{1a}
				2000	Liver		
				300	Kidney		
				250	Fat		
				20	Milk		
Erythromycin	ADI: 0-0.7	Full	66 (2006)	100	Muscle, Liver,	Chicken, Turkey	Erythromycin A
					Kidney, Fat/Skin		
				50	Eggs	Chicken	
Estradiol-17β	ADI: 0-0.05	Full	52 (1999)	Not specified	Muscle, Kidney, Fat	Liver, Cattle	

Substance	ADI/ARfD (µg/kg bw)	ADI Status	JECFA	MRL (µg/kg)	Tissue	Species	Marker residue and other remarks
Flumethrin	ADI: 0-4	Full	85 (2017)	6		Honey	Flumethrin (trans-Z1 and trans Z2 diastereomers at a ratio of approximately 60:40).
Furazolidone	No ADI or ARfD		40 (1992)	No MRL			
Gentamicin	ADI: 0-20	Full	50 (1998)	100	Muscle, Fat	Cattle, Pig	Gentamicin
				2000	Liver		
				5000	Kidney		
				200	Milk	Cattle	
Gentian violet	No ADI or ARfD		78 (2013)	No MRL			
Imidocarb	ADI: 0-10	Full	60 (2003)	300	Muscle		Imidocarb, free base
				1500	Liver	Cattle	
				2000	Kidney		
				50	Fat, Milk		
Iprnidazole	No ADI or ARfD		34 (1989)	No MRL			
Isometamidium	ADI: 0-100	Full	40 (1992)	100	Muscle, Fat, Milk	Cattle	Isometamidium
				500	Liver		
				1000	Kidney		

Substance	ADI/ARfD (µg/kg bw)	ADI Status	JECFA	MRL (µg/kg)	Tissue	Species	Marker residue and other remarks
Ivermectin	ADI: 0–10 ARfD: 200	Full	81 (2015)	30 800 100 400	Muscle Liver Kidney Fat	Cattle Cattle Cattle Cattle	Ivermectin B _{1a} . The Committee considers that the presence of high concentrations of ivermectin residues at the injection site is product dependent and must be assessed on a case-by-case basis during marketing authorization by comparison of suitable acute dietary exposure estimates with the ARfD.
				15 20 10	Liver Fat Milk	Pig, Sheep Pig, Sheep Cattle	
Lasalocid sodium	ADI: 0–5	Full	81 (2015)	400 1200	Muscle Liver	Chicken, Turkey, Quail, Pheasant	
				600 600	Kidney Fat/Skin		
Levamisole	ADI: 0-6	Full	42 (1994)	10 100	Muscle, Kidney, Fat Liver	Cattle, Sheep, Pig, Poultry	
Lincomycin	ADI: 0-30	Full	62 (2004)	200 500	Muscle Liver	Chicken, Pig Chicken, Pig	Lincomycin A separate MRL of 300 µg/kg for skin with

Substance	ADI/ARfD (µg/kg bw)	ADI Status	JECFA	MRL (µg/kg)	Tissue	Species	Marker residue and other remarks
Lufenuron	ADI:0-20	Full	85 (2017)	1500	Kidney	Pig	adhering fat for pigs was recommended in order to reflect the concentrations found in skin of pigs and this MRL was also extended skin/fat for chicken.
				500	Kidney	Chicken	
				100	Fat	Chicken, Pig	
				150	Milk	Cattle	
Lufenuron	ADI:0-20	Full	85 (2017)	1350	Muscle ^b	Finfish ^a	Lufenuron
				1	Muscle	Cattle	Melengestrol acetate
				10	Liver		
				2	Kidney		
Melengestrol Acetate	ADI: 0-0.03	Full	66 (2006)	18	Fat		
				No MRL			
				34 (1989)			
				70 (2008)	Muscle, Liver, Kidney	Chicken, Turkey, Quail	Monensin
Metronidazole	No ADI or ARfD	Full	75 (2011)	10	Muscle, Kidney	Cattle, Sheep, Goat	Cattle liver MRL revised at 75 JECFA
				20	Liver	Sheep, Goat	
				100	Liver	Cattle	
Monensin	ADI: 0-10	Full	75 (2011)	100	Fat	Cattle, Sheep, Goat, Chicken, Turkey, Quail	Cattle liver MRL revised at 75 JECFA
				2	Milk	Cattle	

Substance	ADI/ARFD (µg/kg bw)	ADI Status	JECFA	MRL (µg/kg)	Tissue	Species	Marker residue and other remarks
Monepantel	ADI: 0-20	Full	78 (2013)	500 7000 1700 13000	Muscle Liver Kidney Fat	Sheep	Monepantel sulfone
	ADI: 0-20	Full	85 (2017)	300 2000 1000 7000	Muscle Liver Kidney Fat	Cattle	Monepantel sulfone
Moxidectin	ADI: 0-2	Full	50 (1998)	20 50 100 50 500	Muscle Muscle Liver Kidney Fat	Cattle, Deer Sheep Cattle, Deer, Sheep Cattle, Deer, Sheep Cattle, Deer, Sheep	Moxidectin The Committee noted very high concentrations and great variation in the residue levels at the injection site in cattle over a 49-day period after dosing.
Narasin	ADI: 0-5	Full	70 (2008) 75 (2011)	15 50 15 50	Muscle, Kidney Liver, Fat Muscle, Kidney Liver, Fat	Chicken, Pig Chicken, Pig Cattle Cattle	Narasin A Temporary MRLs for cattle, replaced with full MRLs in cattle tissue
Neomycin	ADI: 0-60	Full	60 (2003)	500 10000 1500 500	Muscle, Fat, Liver Kidney Milk Eggs	Cattle, Chicken, Sheep, Turkey Goat, Pig, Duck Cattle Chicken	Neomycin

Substance	ADI/ARfD (µg/kg bw)	ADI Status	JECFA	MRL (µg/kg)	Tissue	Species	Marker residue and other remarks
Nicarbazin	ADI: 0-400	Full	50 (1998)	200	Muscle, Liver, Kidney, Fat/Skin	Chicken (broilers)	N,N'-bis(4-nitrophenyl)urea
Nitrofurazone/ Nitrofurals	No ADI		40 (1992)	No MRL			
Olaquinox	No ADI or ARfD		42 (1994)	No MRL			The Committee recommended no MRLs but noted that 4µg/kg in muscle of pigs of the metabolite MQCA (3-Methylquinoxaline-2-carboxylic acid) is consistent with Good Veterinary Practice.
Oxendazole (See Febantel)							
Oxolinic acid	No ADI or ARfD		43 (1994)	No MRL			
Oxytetracycline See chlortetracycline							
Permethrin	No ADI or ARfD		54 (2000)	No MRL			
Phoxim	ADI:0-4	Full	62 (2004)	50 400	Muscle, Kidney Fat	Liver, Goat, Pig, Sheep	Phoxim

Substance	ADI/ARFD (µg/kg bw)	ADI Status	JECFA	MRL (µg/kg)	Tissue	Species	Marker residue and other remarks
Pirlimycin	ADI: 0-8	Full	62 (2004)	100 1000	Muscle, Fat Liver	Cattle	Pirlimycin
				400 100	Kidney Milk		
Porcine Somatotropin	ADI: Not Specified		52 (1999)	Not Specified	Muscle, Liver, Kidney, Fat	Pig	
Procaine benzylpenicillin	ADI: < 30µg/person/ day of the penicillin moiety	Full	50 (1998)	50 4	Muscle, Kidney Milk	Liver, All species	Benzylpenicillin
Progesterone	ADI: 0-30	Full	52 (1999)	Not Specified	Muscle, Liver, Kidney, Fat	Cattle	
Propionyl- promazine	No ADI or ARFD		38 (1991)	No MRL			
Ractopamine hydrochloride	ADI: 0-1	Full	66 (2006)	10 40 90	Muscle, Fat Liver Kidney	Cattle, Pig	Ractopamine
Ronidazole	No ADI or ARFD		42 (1994)	No MRL			
Sarafloxacin	ADI: 0-0.3	Full	50 (1998)	10 80 20	Muscle Liver, Kidney Fat/skin	Chicken, Turkey	Sarafloxacin

Substance	ADI/ARfD (µg/kg bw)	ADI Status	JECFA	MRL (µg/kg)	Tissue	Species	Marker residue and other remarks
Spectinomycin	ADI: 0-40	Full	50 (1998)	500	Muscle	Cattle, Chicken, Pig,	Spectinomycin
				2000	Liver, Fat	Sheep	
				5000	Kidney	Chicken	
				2000	Eggs		
				200(µg/L)	Milk	Cattle	
Spiramycin	ADI: 0-50	Full	48 (1997)	200	Muscle	Cattle, Chicken, Pig	For cattle and chicken, MRLs are expressed as the sum of spiramycin and neospiramycin.
				600	Liver	Cattle, Chicken, Pig	
				300	Kidney	Cattle, Pig	
				800	Kidney	Chicken	
				300	Fat	Cattle, Chicken, Pig	
Streptomycin (See dihydro- treptomycin)	ADI: 0-50	Full	42 (1994)	200(µg/L)	Milk	Cattle	For pigs, the MRLs are expressed as spiramycin equivalents (antimicrobial active residues).
Sulfadimidine (Sulfamethazine)	ADI: 0-50	Full	42 (1994)	100	Muscle, Kidney, Fat	Cattle, Sheep, Pig, Poultry	Sulfadimidine
Sulfathiazole	No ADI or ARfD		34 (1989)	No MRL	Milk	Cattle	
Teflubenzuron	ADI: 0-5	Full	81 (2015)	400	Muscle	Salmon	Teflubenzuron
				400	Muscle plus skin in natural proportion	Salmon	

Substance	ADI/ARFD (µg/kg bw)	ADI Status	JECFA	MRL (µg/kg)	Tissue	Species	Marker residue and other remarks
Testosterone	ADI: 0-2	Full	52 (1999)	Not specified	Muscle, Liver, Kidney, Fat	Cattle	
Tetracycline (See chlortetracycline)							
Thiamphenicol	ADI: 0-5	Full	58 (2002)	No MRL			
Tiabendazole (Thiabendazole)	ADI: 0-100	Full	58 (2002)	100	Muscle, Liver, Kidney, Fat	Cattle, Pig, Goat, Sheep	Sum of tiabendazole + 5- hydroxy tiabendazole
				100	Milk	Cattle, Goat	
Tilmicosin	ADI: 0-40	Full	70 (2008)	100	Muscle, Fat	Cattle, Pig, Sheep	Tilmicosin
				1000	Liver	Cattle Sheep	
				1500	Liver	Pig	
				300	Kidney	Cattle, Sheep	
				1000	Kidney	Pig	
				150	Muscle	Chicken	
				100	Muscle	Turkey	
				2400	Liver	Chicken	
				1400	Liver	Turkey	
				600	Kidney	Chicken	
				1200	Kidney	Turkey	
				250	Skin/Fat	Chicken, Turkey	
Trenbolone acetate	ADI: 0-0.02	Full	34 (1989)	2 10	Muscle Liver	Cattle	β Trenbolone for muscle α-Trenbolone for liver

Substance	ADI/ARfD (µg/kg bw)	ADI Status	JECFA	MRL (µg/kg)	Tissue	Species	Marker residue and other remarks
Trichlorfon (Metrifonate)	ADI: 0-2	Full	66(2006)	50	Milk	Cattle	Trichlorfon
				50	Muscle, Liver, Kidney, Fat		Guidance MRLs at the limit of quantitation of the analytical method for monitoring purposes. No residues should be present in tissues when used with Good Veterinary Practice.
Triclabendazole	ADI: 0-3	Full	70 (2008)	250	Muscle	Cattle	Keto-triclabendazole
				850	Liver	Cattle	
				400	Kidney	Cattle	
				200	Muscle	Sheep	
				300	Liver	Sheep	
				200	Kidney	Sheep	
				100	Fat	Sheep, Cattle	
				100	Muscle, Liver,	Cattle, Pig,	
Tylosin	ADI: 0-30	Full	70 (2008)		Kidney	Chicken	Tylosin A
				100	Fat	Cattle, Pig	
				100	Skin/Fat	Chicken	
				100	Milk	Cattle	
				300	Eggs	Chicken	
Xylazine	No ADI or ARfD		47 (996)	No MRL			

Substance	ADI/ARfD (µg/kg bw)	ADI Status	JECFA	MRL (µg/kg)	Tissue	Species	Marker residue and other remarks
Zeranol	ADI: 0-0.5	Full	32 (1987)	2	Muscle	Cattle	Zeranol
				10	Liver		
Zilpateterol hydrochloride	ADI: 0–0.04 ARfD: 0.04	Full	81 (2015)	0.5	Muscle	Cattle	Zilpateterol (free base). The GEADE is 1.9 µg/day for the general population, which represents approximately 80% of the ARfD.
				3.5	Liver		
				3.3	Kidney		

^a The term “finfish” includes all fish species.
^b Muscle plus skin in natural proportion.

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FAO JECFA MONOGRAPHS

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1. Combined compendium of food additive specifications – JECFA specifications monographs from the 1st to the 65th meeting. Vol. 1: Food additives A – D; Vol. 2: Food additives E – O; Vol. 3: Food additives P – Z; Vol. 4: Analytical methods, test procedures and laboratory solutions.
2. Residue evaluation of certain veterinary drugs – Joint FAO/WHO Expert Committee on Food Additives, 66th meeting 2006
3. Compendium of food additive specifications – Joint FAO/WHO Expert Committee on Food Additives, 67th meeting 2006
4. Compendium of food additive specifications – Joint FAO/WHO Expert Committee on Food Additives – 68th meeting 2007
5. Compendium of food additive specifications – Joint FAO/WHO Expert Committee on Food Additives, 69th meeting 2008
6. Residue evaluation of certain veterinary drugs – Joint FAO/WHO Expert Committee on Food Additives. 70th meeting 2008
7. Compendium of food additive specifications – Joint FAO/WHO Expert Committee on Food Additives, 71st meeting 2009
8. Safety evaluation of certain contaminants in food – Joint FAO/WHO Expert Committee on Food Additives, 72nd meeting 2010. Joint FAO/WHO publication: WHO Food Additives Series No. 63/FAO JECFA Monographs 8
9. Residue evaluation of certain veterinary drugs – Joint FAO/WHO Expert Committee on Food Additives, Meeting 2010 – Evaluation of data on ractopamine residues in pig tissues
10. Compendium of food additive specifications – Joint FAO/WHO Expert Committee on Food Additives, 73rd meeting 2010
11. Compendium of food additive specifications – Joint FAO/WHO Expert Committee on Food Additives, 74th meeting 2011
12. Residue evaluation of certain veterinary drugs – Joint FAO/WHO Expert Committee on Food Additives. 75th meeting 2011
13. Compendium of food additive specifications – Joint FAO/WHO Expert Committee on Food Additives, 76th meeting 2012
14. Compendium of food additive specifications – Joint FAO/WHO Expert Committee on Food Additives, 77th meeting 2013
15. Residue evaluation of certain veterinary drugs, Joint FAO/WHO Expert Committee on Food Additives. 78th meeting 2014
16. Compendium of food additive specifications, Joint FAO/WHO Expert Committee on Food Additives. 79th meeting 2014
17. Compendium of food additive specifications, Joint FAO/WHO Expert Committee on Food Additives. 80th meeting 2015
18. Residue Evaluation of Certain Veterinary Drugs; Joint FAO/WHO Expert Committee on Food Additives. 81st meeting 2015.

19. Compendium of Food Additive Specifications; Joint FAO/WHO Expert Committee on Food Additives. 82nd Meeting 2015.

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RESIDUE EVALUATION OF CERTAIN VETERINARY DRUGS

Joint FAO/WHO Expert Committee on Food Additives

85th Meeting 2017

This volume of FAO JECFA Monographs contains residue evaluation of certain veterinary drugs prepared at the 85th Meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), held in Geneva, 17–26 October 2017. This was the twenty-fifth JECFA meeting specifically convened to consider residues of veterinary drugs in food. The Committee elaborated principles for evaluating the safety of residues of veterinary drugs in food, for establishing acceptable daily intakes (ADIs) and acute reference doses (ARfDs) and for recommending maximum residue limits (MRLs) for such residues when the drugs under consideration are administered to food-producing animals in accordance with good practice in the use of veterinary drugs (GVP). Furthermore, the committee evaluated the safety of residues of eight veterinary drugs and responded to specific concerns raised by the Codex Committee on Residues of Veterinary Drugs in Foods. The enclosed monographs provide the scientific basis for the recommendations of MRLs, including information on chemical identity and properties of the compounds, pharmacokinetics and metabolism, residue depletion studies and analytical methods validated and used for the detection and quantification of the compounds. This publication and other documents produced by JECFA contain information that is useful to all those who work with or are involved with recommending or controlling maximum residue limits for veterinary drugs in food.

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