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6

RESIDUE EVALUATION OF CERTAIN VETERINARY DRUGS

Joint FAO/WHO Expert Committee on Food Additives

70th meeting 2008

World Health Organization

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FAO JECFA Monographs 6

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FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS
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SPECIAL NOTE

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Geneva, Switzerland, 21 – 29 October, 2008

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ABBREVIATIONS

ADI	Acceptable daily intake
AOAC	AOAC International
AUC	Area under the curve
BW or bw	Body weight
CAC	Codex Alimentarius Commission
CAS	Chemical Abstracts Service
CCRVDF	Codex Committee on Residues of Veterinary Drugs in Foods
Cl	Clearance rate
Cmax	Maximum concentration
CR	Renal clearance
CV	Coefficient of variation
Cv_r	Repeatability
Cc_R	Reproducibility
ECD	Electron capture detector
EDI	Estimated daily intake
EFSA	European Food Safety Authority
EMEA	European Medicines Agency
FAO	Food and Agriculture Organization of the UN
FDA	US Food and Drug Administration
GC	Gas chromatography
GLP	Good laboratory practice
H or h	Hour
HPLC	High pressure liquid chromatography
IM	Intramuscular
IR	Infrared
IU	International Unit
IUPAC	International Union of Pure and Applied Chemistry
IV	Intravenous
JECFA	Joint FAO/WHO Expert Committee on Food Additives
JMPR	Joint FAO/WHO Meeting on Pesticide Residues
Kg or kg	Kilogram (10^3grams)
LC	Liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantitation
µg	microgram (10^{-6}grams)
mg	milligram (10^{-3}grams)
min	Minimum or minute
MGA	Melengestrol acetate
mL or ml	milliliter
MIC	Minimum Inhibitory Concentration
MRL	Maximum Residue Limit
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MW or mw	Molecular weight
N	Negative
NA or na	Not analyzed or not applicable
NADA	New Animal Drug Application
NC or nc	Not calculated
ND	Not detected
NICI	Negative ion chemical ionization
NOEL	No effect level

NQ	Non quantifiable
P	Positive
QA	Quality assurance
QC	Quality control
RfD	Acute reference dose
RP	Reverse phase
SC	Subcutaneous (injection)
SD	Standard deviation
S/N	Signal to noise ratio
SPE	Solid phase extraction
SD	Standard deviation
s.e.	Standard error
$t_{½}$	Half life
TR	Total residue
TLC	Thin layer chromatography
TMDI	Theoretical maximum daily intake
TRR	Total radiolabelled residues
TRS	Technical Report Series
TSP	Thermospray
USP	United States Pharmacopoeia
UV	Ultraviolet
Vd	Volume of distribution
WHO	World Health Organization

INTRODUCTION

The monographs in this volume of the FAO JECFA Monographs on the residues of, statements on, or other parameters of the veterinary drugs on the agenda were prepared by the invited experts for the seventieth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) that was held in Geneva, Switzerland, 21-29 October, 2008. This was the eighteenth meeting of JECFA convened specifically to consider residues of veterinary drugs in food producing animal species. The Committee has evaluated residues of veterinary drugs at its 12th, 26th, 27th, 32nd, 34th, 36th, 38th, 40th, 42nd, 43rd, 45th, 47th, 48th, 50th, 52nd, 54th, 58th, 60th, 62nd and 66th meetings (ref. 1-15 and 18-22). 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 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.

There is an important feature to bring to the attention of readers. This volume of the FAO JECFA Monographs is the second in a new format for the presentation of monographs from meetings of the Committee. It was also the second meeting of JECFA subsequent to the completion of the workshop to update the principles and methods of risk assessment for MRLs for pesticides and veterinary drugs, held jointly by FAO/RIVM/WHO, in Bilthoven, The Netherlands, 7 - 11 November, 2005 (ref. 23). Specifically, the Committee continued to implement some of the more significant recommendations in the workshop report, including the concept of using median residue values to estimate daily intakes of residues of veterinary drugs in food for chronic exposure intake estimates and to consider a specific approach for recommending MRLs for substances used in apiculture.

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, November 1984 (ref. 16). 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 (ref. 17). 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. Seventeen such meetings of JECFA have been held prior to this meeting of JECFA.

70th Meeting of JECFA

The present volume, in the new format, contains monographs of the residue data on eight of the substances scheduled for evaluation at the 70th meeting of the Committee. One substance, melengestrol acetate, was for review of toxicological data only and no residue monograph was prepared. In addition, a discussion paper and recommendations to CCRVDF was elaborated regarding specific approaches for MRLs in honey. The Committee was also asked to comment on use of malachite green in aquaculture. 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 70th meeting is found in Annex 2. **The monographs and general considerations on risk assessment principles of this**

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

On-line edition of Residues of some veterinary drugs in animals and foods (from FAO JECFA Monographs and FAO Food and Nutrition paper Number 41)

The monographs and statements that have been published in the FAO JECFA Monographs 2 and this volume as well as those published in FAO Food and Nutrition Paper Series 41 (sixteen volumes since 1988) are all available online at http://www.fao.org/ag/agn/jecfa-vetdrugs/search.html. 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 the FAO homepage of JECFA at http://www.fao.org/ag/agn/agns/jecfa_index_en.asp . Readers are invited to address comments and questions on this publication and other topics related to the work of JECFA to:

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RESIDUES OF VETERINARY DRUGS IN HONEY AND POSSIBLE APPROACHES TO DERIVE MRLS FOR THIS COMMODITY

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The 66th meeting of the Committee discussed the report of the 2005 meeting of the Joint FAO/RIVM/WHO workshop "Updating the Principles and Methods of Risk Assessment: Maximum Residue Levels (MRLs) for Pesticides and Veterinary Drugs" (FAO/WHO, 2005). With regard to the workshop recommendation on honey, that JECFA should investigate a specific approach for MRLs in honey and prepare a paper to consider if a separate approach for honey is warranted, the 66th meeting of the Committee agreed that a paper should be prepared for consideration at the next meeting of the Committee devoted to residues of veterinary drugs in food. The paper should consider the relevant scientific issues specific to honey, and accordingly, develop draft recommendations for consideration by the Committee and by CCRVDF. It should be noted that the 52nd meeting of the Committee (FAO/WHO, 2000) first considered the subject and requested that the 12th Session of the CCRVDF comment on the matter. The report of the 12th Session of the CCRVDF does not include any comments on the matter.

There is substantial production and trade in honey worldwide; however, there are very limited numbers of standards for residues (MRL) in honey. Table 1 shows five year honey production figures for the different parts of the world. Asia is the greatest honey producer. Other major honey producing regions or countries are the European Union, the United States and Argentina. Honey production does naturally vary from year to year depending on all manner of environmental factors such as cropping or the weather and the impact of pests and pathogens.

Table 1: World honey production by region 2001-2005 (1000t)

	2001	2002	2003	2004	2005
Africa	145	153	152	152	154
North and Central America	193	188	191	187	188
South America	127	132	137	133	133
Asia	458	497	525	543	545
Europe	311	294	320	328	332
Oceania	29	23	29	29	29
Total	1,264	1,287	1,354	1,372	1,381

Source FAO

A list of products used world-wide, including the active ingredient where it is available is included in Table 2. ADIs established either by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) or the Joint FAO/WHO Expert Committee on Food Additives (JECFA) are indicated in the table.

Table 2: List of products used in apiculture

Substance	Proprietary product	ADI [mg/kg bw/day]	
		JECFA	JMPR
Acrinathrine	Yes		
Amitraz	Yes		0-0.01
Bromopropylate	Yes		0-0.03
Chlorobenzilate	No		0-0.02
Chlortetracycline	No	0-0.003	
Coumaphos	Yes		[2]
Cymiazole hydrochloride	Yes		
Enilconazol (imazalil)	No		0-0.03
Erythromycin	No	0-0.0007	
Fenproximate	Yes		
Fipronil	No		0-0.0002
Flumethrin	Yes		0-0.004
Formic acid[1]	Yes	0-3	
Fumagillin	Yes		
Lactic acid[1]	No	Not limited	
Lincomycin hydrochloride	?	0-0.03	
Malathion	No		0-0.3
Menthol[1]	Yes	0-4	
Methyl bromide	No		
Monensin	No	0-0.01	
Oxalic acid[1]	Yes		
Oxytetracycline	Yes	0-0.003	
Paradichlorobenzene	No		
Permethrin	yes		0-0.05
Propargite	?		0-0.01
Rifampicin	No		
Spinosad	No		0-0.02
Streptomycin/ dihydrostreptomycin	No	0-0.05	
Sulfathiazole	No	No ADI allocated	
Tau-Fluvalinate	Yes		
Thymol[1]	Yes	Acceptable	
Tylosin tartrate	Yes	0-0.03	

[1]. Substances considered by many national authorities as generally recognized as safe
[2]. Temporary ADI withdrawn in 1980; no ADI allocated in 1990

Points to consider

Several factors have to be considered in developing a process to address the need for recommending MRLs pertaining to the use of veterinary drugs and pesticides for bee health and honey production submitted to the Committee for evaluation. They include but may not be limited to the following points:

- The recommendations must be within the Committee's terms of reference with adequate flexibility to meet differing conditions and availability of information.

- There is a need to accommodate a robust yet conservative approach to facilitate MRL recommendations and encourage sponsorship for studies that the Committee considers necessary for recommending MRLs.

- The drug is available as a commercial product, and the commercial product containing the active ingredient is currently registered by a national or regional authority.

- Honey production and honey bees in most countries are considered as a minor use and/or minor species food product, and the availability of active sponsors to provide studies suitable for recommendations on MRLs is likely to be limited.

- Honey is widely used as a sweetener and glazing agent in confectionary products, breakfast cereals and baked goods, in addition to direct consumption of liquid and set honey, and these uses must be accounted for in intake estimates.

- Several substances use to manage bee health are unlikely to raise public health concern, because intake of residues resulting from effective use are far below internationally established ADIs.

- Some proprietary products are not registered for use in bee colonies, and therefore approved dosages and conditions of use do not exist.

- For a number of substances registered for use by national authorities, no contemporary toxicological evaluation may have been performed, or the review did not result in the establishment of a health-based guidance value, such as an ADI.

- The main groups of substances that typically leave residues in edible bee products are antibiotics (residues in honey and royal jelly) and persistent lipophilic acaricides (residues in wax and propolis).

- Royal jelly should be the subject of a separate and later consideration.

- Residues in both honey and wax need to be considered in exposure estimates. The ratio honey to wax is typically 9:1.

Substances with an ADI and/or MRL in a food producing animal or food commodity

The main groups of substances which typically leave residues in edible bee products are antibiotics (residues in honey and royal jelly) and persistent lipophilic acaricides (residues in wax and propolis). Of the products known to be used for treatment of bee diseases listed in Table 2, most, but not all have a national registration, a JECFA or JMPR evaluation with an ADI and/or MRL, or the equivalent, in national legislation, for either a food producing animal or other food commodity, and usually the active ingredients are substances with a long history of use.

Where an established ADI or MRL exists for use in other species as either a veterinary medicine or pesticide, application to a minor use/minor species (e.g., bee and honey products) would generally require a smaller set of additional data as an ADI (or MRL) exists in a generally recognized major food producing animal or bird and their products (e.g., milk and eggs). A critical issue, however, are the studies necessary to provide the relevant data. Because of the complexity of honey production by bees, this may be difficult, as discussed below.

Veterinary drugs (or pesticides) for apiculture use submitted to the JECFA for evaluation should meet general criteria for evaluation, including that the use of the drug will result in drug residues in honey and other edible products obtained from bees, that may constitute a potential public health concern and/or cause impediments in trade. The submitted dossier should include confirmation of

authorization and a precise description of approved dosages and conditions of use. The dossier ought to include data suitable for the establishment of a health based guidance value (for substances yet without an international established ADI) and for the evaluation of residues including the recommendation of MRLs.

Substances generally regarded as safe

Several substances are unlikely to raise public health concerns because any use in food producing animals or especially the use in bees is generally regarded as safe. Examples of such substances include formic acid, lactic acid, oxalic acid, thymol and menthol. In the case of a substance that has clear documentation to support the designation as "generally regarded as safe" by national regulatory authorities and not requiring a MRL, a similar designation can be made. It would require a proviso that equivalence can be demonstrated in honey and that the ADI is sufficient so that no MRL should be required and the ADI is not exceeded. In the case of a new substance not previously considered for registration by national authorities, substances would have to be evaluated as new animal drugs or pesticides and subject to a full food safety risk assessment.

Use of non-approved veterinary drugs or pesticides

In the situation where a substance is not approved for use in food producing animals (e.g., chloramphenicol or nitrofurans), no exception for honey would be applied.

Suggested tools for data generation

Account must be taken of the unique nature of honey and how the residue behaves in honey as well as the numerous factors noted above. It should be noted that all the drug residues and metabolites collect in the honey and the only mechanisms for reduction are dilution as more honey is produced or removed from the hive, photochemical or thermal degradation of the residues in the honey or through such factors as pH and environmental conditions.

The biological variability of residue concentrations found under seemingly the same conditions in a trial or under similar conditions in different trials may be very high, as bees commonly move honey around the hive as required, and this can lead to significant variation in residue concentration even across the same frame in all three hive dimensions. This suggests the need for trials to be conducted over more than one honey producing season to take account of seasonal variability. Likewise, a number of active substances are not stable in honey.

Residue Study Design

For substances leaving residues in honey and related products, well designed residue trials under the established practical conditions of use should be conducted. The trials should preferably be performed under GLP compliant conditions and use of data from non-compliant studies would need to be justified. Design criteria for residue data studies should include the following considerations:

- the number of apiaries involved representing a variety of honey types;
- number of hives per apiary sampled;
- number of frames per hive sampled;
- number of samples of wax and honey to be taken from a frame;
- number and spacing of time points to describe the kinetics of formation and depletion of honey in the edible products;

- estimates of amounts of surplus honey present at the beginning, during and after the treatment until the end of the trial;
- scheme for the analysis of individual and bulk samples;
- climatic information for the duration of the trial including season of the year (e.g. rainfall);
- crop on which bees forage;
- temperature profile within the hive;
- data on honey flow periodicity;
- data on any supplemental feed given to bees;
- data on bee health and bee/parasite mortality during the study
- a protocol for the analysis of individual and bulk samples
- studies on storage stability of residues in honey.
- analytical methods should be suitable for the purpose and validated in the different matrices (honey, wax, etc.)

Should the Committee make a recommendation on sampling parameters, it should include something comparable to a minimum of 10 samples each of 20 g taken from random positions within one hive.

The quality of the data should allow a statistical evaluation to determine the confidence intervals necessary to recommend the setting of MRLs. The data should show with 95% statistical confidence that 95% of all honey samples from treated bees would be below the MRL and that the estimated intake of residues (considering all other sources of intake) remains below the ADI. As the design of the study depends on many factors, it has to be developed on a product by product basis, depending on the use pattern.

Marker residue

The marker residue concept may not be normally or easily applied in honey scenarios. However, it is important to sufficiently identify and, where feasible, quantify metabolites and degradation products in honey. If a marker residue is proposed and it can be demonstrated that it is appropriate it may be a practical consideration. Analysis of data in shown in Annex 2 on products used in honey bees indicates it may not work in all cases – it could work with amitraz as all residues are hydrolyzed to a single substance for residue analysis. For tylosin it may not, as one of the tylosin metabolites also has antimicrobial activity and it would require the analysis of both microbiologically active substances for exposure considerations (Annex 2 provides more detailed explanations). The ratio of marker to total residue concentrations needs to be established for the whole time period from drug application to the end of the withdrawal time for each matrix.

Dietary intake considerations

The internationally accepted daily dietary intake of honey is generally taken to be 20 g per person per day, as per the JECFA model diet. A review of the WHO GEMS Food 13 cluster diets indicates that median daily intakes are equal to or less than 2 grams per day. These data have been generated based on the FAO food balance sheets and may underestimate consumption by honey eaters. In discussing the criteria for the establishment of an estimate of chronic intake, the Committee concluded that such a figure should be derived from consumption data for “chronic” honey eaters. A number of countries have data specific to consumption in their areas that may be very useful for national standards.

Adequate information regarding how dietary surveys have been conducted is necessary to properly assess dietary consumption figures. Comprehensive intake data are available from the UK on honey consumption across the population and information on key groups is given in the table below. The mean consumption for these groups is within the 20 g per person per day in the standard JECFA diet. However, there are extreme consumers with a chronic exposure in excess of this figure. Table 3 does not show ingestion on a bodyweight basis and this figure will be higher for children and infants.

Table 3: UK consumption of honey in 2000 (UK Food Standards Agency data)

Consumer group	Exposure type	Mean consumption (g/day)	97.5 Percentile consumption (g/day)	Maximum consumption (g/day)
Adult	Acute	7.8	41.2	152
Adult	Chronic	2.4	15.0	77.1
Free living elderly adult	Acute	7.8	42.0	115
Free living adult	Chronic	4.7	32.2	97.8
School children	Acute	4.9	26.4	90
Infant	Acute	2.6	16	64
Infant	Chronic	1.0	7.0	45.1

Explanation**:** Chronic = the amount consumed by individuals over 7 days computed as the average for 1 day. Acute = the highest days consumption for each individual in the survey. Mean Consumer = the total consumption in grams of the food divided by the number of consumers of that food in the survey

The Committee used a study conducted in 1986-1989 in Germany as the basis for the study of methodological aspects of deducing a figure for daily intake of honey, because consumption data were available for more than 9000 consumers of honey. Germany has one of the largest populations of honey consumers in Europe. The data set includes records on more than 23,000 individuals (approximately 47% ≥ 14 years old). Of the more than 23,000 individuals, about 9,000 consumed honey at least once a day. In this data set, the median portion consumed was 18 g per person with a range of 0.1 to 222 g/day. The total consumption of the people over the whole observation period was from 0.54 to 576 g. The results are given in the tables below.

Table 4: Statistics of the individual portions and of the daily intakes

Number of portions:	25730	Number of people:	9019
	Size of portion [g]		Daily intake[1]
Min	0.1	Min	0.08
Max	222	Max	82.29
Median	18	Median	5.57
P90	40	P90	17.14
P95	48	P95	23.44
P97.5	57	P97.5	29.08
P99	75	P99	37.99
P99.9	120	P99.9	67.07
P99.95	144	P99.95	75.02
P99.99	207	P99.99	81.51

1. Averaged over 7 days observation period

The data in Table 4 indicate that for a high percentile portion, suitable for use as an estimation of acute dietary intake, consumption is between 100-150g. Table 4 indicates the current JECFA intake

value is between the 90th and 95th percentile of daily intakes. The median daily intake of all eaters (Table 4) is below the current dietary consumption of 20 g/day. The data provided by the UK and Germany suggest that an acute dietary consumption factor needs to be determined by the Committee.

Table 5: Statistics of the average portions consumed by a person (only days where honey is consumed)

Portions of honey consumed in 7 days:	1	2	3	4	5	6	7	8	9 to 12
Number of people	2983	1886	1374	914	693	571	521	47	30
	Average size of a portion [g]								
Min	0.5	0.5	1.2	2.0	2.3	0.8	4.5	7.5	3.6
Max	144.0	85.0	96.0	98.0	80.8	96.0	81.4	58.8	30.0
Median	18.0	16.5	17.2	16.0	16.2	17.3	18.0	14.8	14.9
P95	48.0	45.0	44.0	40.5	39.6	40.0	47.6	Sample size too small for meaningful calculations	
P97.5	54.0	50.0	54.0	46.6	47.9	48.1	55.3		
P99	72.0	60.0	65.5	53.8	52.2	60.0	67.3		
P99.9	100.0	80.5	90.3	86.1	68.2	89.2	81.0		

Table 6: Statistics of the averaged daily intakes of a person (total amounts consumed averaged over 7 days)

Portions of honey consumed in 7 days:	1	2	3	4	5	6	7	8	9 to 12
Number of people	2983	1886	1374	914	693	571	521	47	30
	Daily consumption [g]/person								
Min	0.08	0.15	0.53	1.17	1.66	0.73	4.50	8.57	5.15
Max	20.57	24.29	41.14	56.00	57.71	82.29	81.43	67.14	38.57
Median	2.57	4.71	7.36	9.14	11.57	14.86	18.00	16.86	20.27
P95	6.86	12.86	18.86	23.14	28.29	34.29	47.57	Sample size too small for meaningful calculations	
P97.5	7.71	14.29	23.14	26.65	34.22	41.25	55.29		
P99	10.29	17.14	28.05	30.75	37.26	51.43	67.29		
P99.9	14.29	22.99	38.70	49.22	48.72	76.42	80.98		

Additional data to collaborate the tables and discussions above is provided in Annex 1.

RECOMMENDATIONS

In considering the matters of interest noted in this report and the complex and unique nature of honey and honey bees, the Committee may not be able to take any specific approaches without further guidance from CCRVDF. The Committee therefore made the following recommendations to CCRVDF:

1. That CCRVDF with the aid of member countries compile a comprehensive list of all veterinary drugs registered for honey production and bee health and develop a priority list of veterinary drugs for use in honey bees to be considered for risk assessment by JECFA.

2. That CCRVDF and member countries be encouraged to provide data on honey consumption, considering both direct and indirect honey intake, for purposes of improved intake assessments as part of the risk assessment for recommending MRLs.

3. That CCRVDF consider extension of good veterinary practice guidelines to include honey production.

4. That the CCRVDF ad hoc Working Group on Methods of Analysis and Sampling consider analytical methods for residues in honey.

5. That the CCRVDF provide guidance on the appropriate percentile for an estimation of acute intake.

The Committee further makes the following recommendation to the JECFA Joint Secretariat:

1. That the JMPR Joint Secretariat be advised of the Committee's report regarding residues in honey and considerations of residues from use of pesticides in honey production and bee health.

REFERENCES

FAO/WHO (2000). Evaluation of Certain Veterinary Drug Residues in Foods (Fifty-second Report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series No. 893.

FAO/WHO (2005). Final report from Workshop on Update of Principles and Methods of Risk Assessment – MRLs for pesticides and veterinary drugs. FAO/RIVM/WHO, Bilthoven, the Netherlands, 7-11 November, 2005. Available at the FAO JECFA website at: ftp://ftp.fao.org/ag/agn/jecfa/bilthoven_2005.pdf .

ANNEX 1

Supporting Documentation - Honey Consumption

The Committee reviewed the adequacy for the estimation of acute and chronic intakes of its currently used consumption figure of 20 g honey per person per day. When the original food basket was established at early meetings of the Committee and at the first sessions of the CCRVDF care was taken that the consumption figures protected the preferential eater of foods of animal origin. With the data available in the late 1980's, the approximately 97.5th percentile of daily consumption by the consumers of a commodity from a country with a known high consumption of honey was chosen. It was furthermore considered necessary to derive figures which would also cover the intake resulting from consumption of processed products containing the raw commodity. There was concern that the consumption figure for honey did not meet the above criteria.

For the review at the seventieth meeting of the Committee, very limited non-aggregated recent consumption data was available in the public domain. However, the Committee considered as a basis for studying the methodological aspects to find an appropriate honey intake a study conducted in 1986-1989 in Germany (NVS 1989), because from this study non-aggregated consumption data of more than 9000 consumers of honey were available in an electronic public use file. Additional information from the United Kingdom, the Netherlands and Germany was made available by experts participating in the meeting.

Germany is one country with high honey consumption. Of the 23209 individuals (10985 $\geq$ 14 years old) delivering valid data in the above mentioned study, 9019 had consumed honey at least once on one day of the 7 day observation period. For a few persons data for more than 7 days were available. A total of 25730 portions were consumed with a median of 18 g per portion and a range of 0.1 to 222 g per portion. The total consumption of the participants over the whole observation period was from 0.54 to 576 g and was slightly higher for males compared to females (for example, in seven days the following total amounts were consumed: median for 3845 males 42 g, median for 4753 females 36 g; 97.5th percentile for males 225g, 97.5th percentile for females 189 g). When consumption was compared in relation to body weight of the participants consumption was slightly higher for females (for example, in seven days the following total amounts were consumed on a body weight basis: median for 3845 males 0.253 g/kg bw, median for 4753 females 0.268 g/kg bw; 97.5th percentile for males 0.952 g/kg bw, 97.5th percentile for females 0.968 g/kg bw). Some persons consumed up to 12 portions during the observation time, which means 2 portions on several days; however, the median consumption frequency was 2 portions in seven days. A small subpopulation of individuals ate honey every day. The results of an initial statistical evaluation are given in the tables below.

The statistics of the individual portions (left part of table 1) could serve as a basis for the determination of the *"acute intake"*. The acute intake represents a *high single intake* or a *high amount consumed over a short period of time*, such as one day. Since only a few people consumed honey more than once a day, a statistics of the 25580 individual daily intakes would yield almost exactly the same values for the median and higher percentiles as the above statistics of portion sizes. Thus, the left part of table 1 can be directly used to determine an appropriate figure for an estimated acute intake. The right part of table 1, however, is not suitable to estimate the "chronic intake". Most persons consumed honey only twice in seven days, and this cannot be considered chronic intake.

Table 1: Statistics of the individual consumed honey portions and daily intakes

Number of portions:	25730	Number of persons:	9019
	Portion size [g]		Daily intake*[g]
Min	0.1	Min	0.08
Max	222	Max	82.29
Median	18	Median	5.57
P90	40	P90	17.14
P95	48	P95	23.44
P97.5	57	P97.5	29.08
P99	75	P99	37.99
P99.9	120	P99.9	67.07
P99.95	144	P99.95	75.02
P99.99	207	P99.99	81.51

*averaged over the whole observation period

Therefore, it was necessary to look at the subpopulation of individuals who consume honey more regularly in order to obtain a suitable estimate of chronic intake. Such an approach is discussed on the basis of the following two tables 2 and 3. In table 2, it is investigated whether or not the portion size of those who consumed honey occasionally and of those who consumed it more regularly is similar. The 9019 consumers were grouped into nine groups according to the number of portions they ate during the observation period. The results obtained for the first seven groups are shown in the tables. Intake was calculated as total amount consumed divided by the number of portions eaten. It can be seen, that the statistics of the median and of the higher percentiles of the average portions sizes are more or less similar for all groups. This means that - if some people consumed more honey than others – this was primarily due to higher frequencies of consumption.

Table 2: Statistics of the averaged daily amounts consumed by a person
(Average calculated for the days where the persons consumed honey)

Portions of honey consumed in 7 days:	1	2	3	4	5	6	7
Number of persons	2983	1886	1374	914	693	571	521
	Daily consumption [g]/person						
Min	0.5	0.5	1.2	2.0	2.3	0.8	4.5
Max	144.0	85.0	96.0	98.0	80.8	96.0	81.4
Median	18.0	16.5	17.2	16.0	16.2	17.3	18.0
P95	48.0	45.0	44.0	40.5	39.6	40.0	47.6
P97.5	54.0	50.0	54.0	46.6	47.9	48.1	55.3
P99	72.0	60.0	65.5	53.8	52.2	60.0	67.3
P99.9	100.0	80.5	90.3	86.1	68.2	89.2	81.0

The median portion size is more or less independent on the frequency of honey consumption.

However, if one now calculates (see table 3) the daily intakes for the same groups and consumption averaged over the 7 days observation period, the median intake and the higher percentiles increase from the group with the lower consumption frequencies to those with higher consumption frequencies. Compared with the right part of table 1 giving an overall median of 5.57 g/person/day one now obtains a median of 18.0 g/person/day for the 521 individuals who consumed honey seven times during seven days. This finding is trivial, but typically ignored in the evaluation of many studies. Column 7 is identical in both tables 2 and 3. The values of column 7 of table 3 represent the best estimate of chronic intakes which can be obtained from the data of this study. Nearly the same numerical values are obtained if the total consumption of the individual persons is divided by the number of the corresponding days on which honey was consumed. If this is done for all 9019 consumers the median, 95th and 97.5th percentiles are 17.8, 48, and 55.3 g per person and day, respectively.

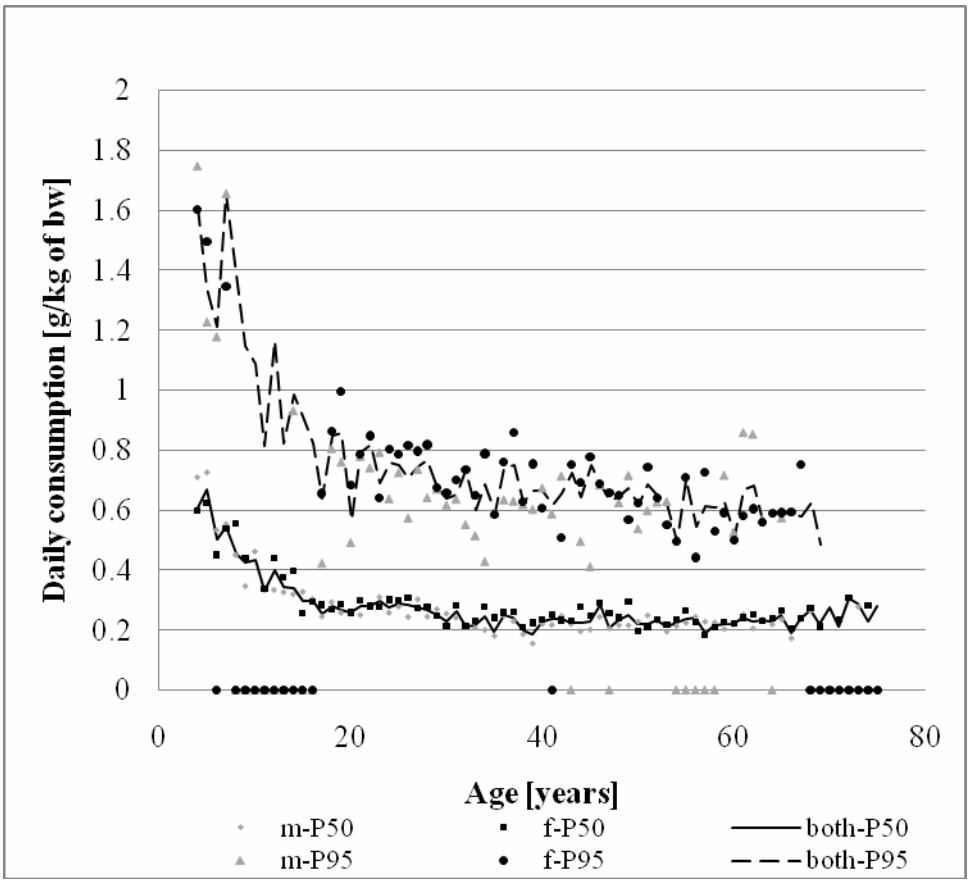
Table 3: Statistics of the averaged daily amounts consumed by a person
(Average calculated from total consumption of a person divided by 7)

Portions of honey consumed in 7 days:	1	2	3	4	5	6	7
Number of persons	2983	1886	1374	914	693	571	521
	Daily consumption [g]/person						
Min	0.08	0.15	0.53	1.17	1.66	0.73	4.50
Max	20.57	24.29	41.14	56.00	57.71	82.29	81.43
Median	2.57	4.71	7.36	9.14	11.57	14.86	18.00
P95	6.86	12.86	18.86	23.14	28.29	34.29	47.57
P97.5	7.71	14.29	23.14	26.65	34.22	41.25	55.29
P99	10.29	17.14	28.05	30.75	37.26	51.43	67.29
P99.9	14.29	22.99	38.70	49.22	48.72	76.42	80.98

Some studies (including the more recent UK study discussed below) found that infants and young children have a significantly higher daily intake in relation to their body weights than adults. In order to investigate this finding, on the basis of the data from the study in Germany, the consumers were grouped according to sex and age (from 4 years to 80 years in steps of one year), and a separate group for the individuals >80 years). For each person the total consumption was divided by the number of days on which honey was consumed and by the body weight. Body weights were available only for 3845 males and 4753 females. Sample sizes for individual age groups were ranging from 9-92 for males, 12-104 for females and 21-196 for both sexes combined. If the size of an age subgroup was $\geq$ 30, a median of the consumption, expressed as g honey/kg of bw/day was calculated; in cases where the group size was $\geq$ 50, a 95th percentile was also estimated.

The results are given in the figure 1, separately for each sex and for both sexes combined. For those age groups with less than the required sample sizes for the calculations a symbol is placed on the zero consumption line representing a missing value. There were sufficient age groups eligible to perform the calculations and to establish a trend. The figure clearly indicates that there is a sharp decline in honey consumption per kg of body weight during the first years of life.

Figure 1: Consumption of honey, expressed in g/kg bw as function of age

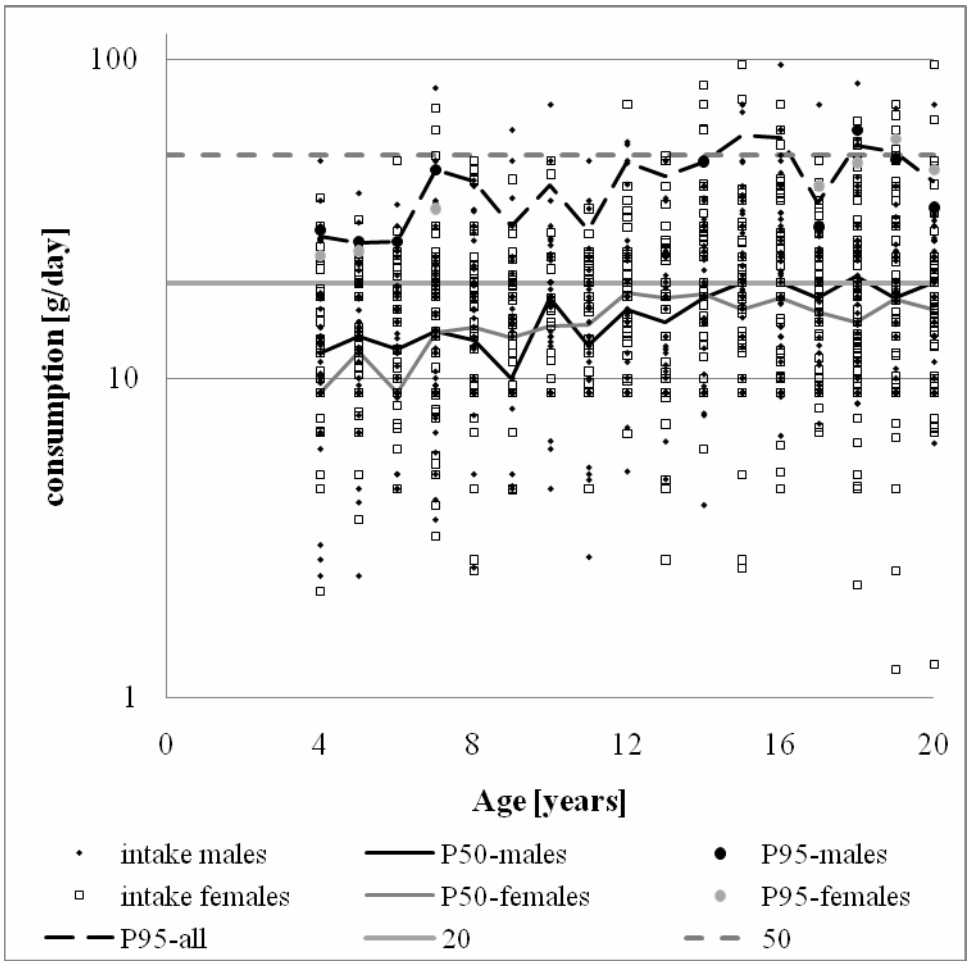


m: males, f: females, P50: 50th percentile, P95: 95th percentile

It was important to find out whether this decline is due to decreasing consumption, increasing body weight or to both influencing factors. Therefore the figure 2 shows the absolute amounts of daily intakes as function of age for consumer between the ages of four years and twenty years. Three of 8698 available data points have been omitted from figure 2 (consumption < 1 gram/day) in order to improve the format of presentation.

For the purpose of figure 2 the total consumption reported for the 7 day reporting period was divided by the number of days on which honey was consumed. The figure shows that both the median and the 95th percentile of daily intakes increase moderately over the first years of life. Therefore, an intake figure which is properly selected from the previously shown statistics for the calculation of JECFA EDIs would be applicable to all age groups. The decrease in consumption expressed on a body weight basis is primarily attributable to increasing body weights.

Figure 2: Variation of median and 95th percentile intakes of honey as function of age.



More recently the Food Standards Agency of the United Kingdom (FSA-UK) carried out a survey yielding also data on honey consumption (National Dietary and Nutrition Survey). Aggregated data were available for the groups defined in table 4.

Table 4: Group design of the FSA study

Group	Type	Explanation
Adults	Chronic	The amount consumed by individuals over 7 days then divided to get the average for 1 day.
Free living elderly		
Infants		
Adults	Acute	The amount consumed on the highest day's consumption for each individual in the survey.
Free living elderly		
Infants		

Except for the chronic intakes of infants all data were expressed in gram per day as well as in gram per kg of body weight per day. The results are summarised in tables 5 a-d.

Table 5a: Acute intake of honey in the FSA study

	Consumer Group		
	Adults	Free living elderly	Infants
Participants	1724	1275	488
Consumers	747	464	216
	Intake [g/day]		
min	0	0	0.001
median	2.973	2.123	0.609
P97.5	41.197	41.985	16
P99	62.313	71.852	26.72
max	152	115	64

The ratios consumers/participants are very similar to those observed in the German study (around 0.4). A direct comparison with the German data is not possible. However, the median "acute" intake of adults seems to be very much lower and the higher percentiles are only moderately lower in the FSA study compared to the German study.

Table 5b: Chronic intake of honey in the FSA study

	Consumer Group		
	Adults	Free living elderly	Infants
Participants	1724	1275	488
Consumers	747	464	216
	Intake [g/day]		
min	0	0	0
median	0.711	0.838	0.113
P97.5	15.006	32.204	6.971
P99	24.849	52.695	14.48
max	77.143	97.75	45.145

The estimated median chronic intakes are extremely low. It is not known whether the individuals participating in this survey consumed honey regularly. The higher percentiles are similar in both studies. A possible explanation could be that the lower percentiles are heavily influenced by the data of the occasional eaters. For the higher percentiles, the influence of the data for the people who eat honey every day is significant.

Table 5c: Acute intake of honey, in relation to body weight in the FSA study

	Consumer Group		
	Adults	Free living elderly	Infants
Participants	1724	1275	488
Consumers	747	464	216
	Intake [g/kg bw/day]		
min	0	0	0
median	0.034	0.022	0.089
P97.5	0.6924	0.71	2.734
P99	0.918	0.924	4.774
max	1.751	1.649	10.936

Again the estimated median values are extremely low; the higher percentiles are not too different from the data in the study from Germany. The data for infants cannot be directly compared, since the youngest children in the German study were 4 years old; however, if one compares with figure 1, the results of the FSA survey seem to qualitatively confirm the trend calculated for the data from Germany.

Table 5d: Chronic intake of honey, in relation to body weight in the FSA study

	Consumer Group		
	Adults	Free living elderly	Infants
Participants	1724	1275	
Consumers	747	464	
	Intake [g/kg bw/day]		
min	0	0	
median	0.008	0.009	
P97.5	0.218	0.53	
P99	0.361	0.707	
max	0.889	1.099	

For the German data, the factor between 97.5th percentile and median is typically about 3-5, depending on the type of statistics, and for the 99th percentile and 97.5th percentile about 1.3. For the FSA data the 97.5th percentile is 14 to 62 times the median and the 99th percentile is 1.3 to 1.8 times the 97.5th percentile.

Data from a survey in the Netherlands (Dutch Food Consumption Survey) had been evaluated. In the Dutch Food Consumption Survey 1997/1998 (VCP3), 6250 respondents registered their food consumption on two consecutive days. 324 respondents (5%) indicated to have consumed honey on at least one of the two survey days. From this it can be concluded that at least 5% of the Dutch population eats honey on a regular basis, for it may be possible that other respondents ate honey, but just not on the survey days. A somewhat more refined estimation is as follows: 251 respondents indicated to have consumed honey on day 1, and 223 respondents on day 2. On average this would mean that on a random day 3.8% (237/6250 x 100%) of the population eats honey. From this it may be concluded that:

- Honey is consumed by about 5% of the population.
- The average intake of Dutch population is 0.7 grams per day.
- Honey consumers (5% of population) consume on average 13g honey per day;
- High consumers (95th percentile of honey consumers) have an intake of 30g of honey per day.

A recent German survey (Banasiak, et al. 2005) found that the 97.5th percentile of honey consumption by children of the age range of 2 up to 5 years of age was 22.1 g/child/day.

In discussing criteria for the establishing an estimate of chronic intake, the Committee concluded that such a figure should be derived from the consumption data of the "chronic" eaters only. In the study from Germany, the 97.5th percentile of consumption by the subgroup consuming $\geq$7 portions in a week was approximately 55 g per consumer per day. The data from the UK were also based on a 7 day survey. Median intakes were low; however, the highest estimated percentiles were not too different from the data from Germany. Data from a 2 day survey in the Netherlands had also been evaluated and the obtained results were significantly lower than estimates based on data from UK and

Germany. Since the data from the UK clearly indicated that on a body weight basis infants and young children have the highest consumption this finding was further investigated and the Committee concluded that this was mainly due to the lower body weight of this group and not to higher intake. In this context a recent German survey found that the 97.5th percentile of honey consumption by children of the age range of 2 up to 5 years of age was 22.1 g/day. The Committee concluded that a consumption figure of 50 g/person and day would be expected to protect all groups of consumers; however, further data are necessary to determine whether this figure also sufficiently covers the consumption of products containing honey.

Honey combs with their original honey content are consumed by a subgroup of consumers. Many lipophilic substances used as acaricides accumulate in wax. Therefore, the labels of certain registered products warn that wax from bees treated with the product should not be consumed. The Committee concluded that in cases where honey combs can be safely consumed it would use a ratio of 9:1 for honey and wax in the estimation of intakes.

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National Dietary and Nutrition Survey. The results of this survey are published in five volumes issued 2002-2004. Links to the reports can be found on the web site of the Food Standards Agency under: http://www.food.gov.uk/science/dietarysurveys/ndnsdocuments/
The information contained in this monograph was communicated by Dr. Jack Kay.

Dutch Food Consumption Survey. A summary of the results was prepared by Polly Boon (RIKILT) and Martine Bakker (RIVM) and communicated through Ir. Astrid S. Bulder.

Banasiak, U., Heseker, H., Sieke, C., Sommerfeld, C., and Vohmann, C. (2005). Abschätzung der Aufnahme von Pflanzenschutzmittel-Rückständen in der Nahrung mit neuen Verzehrsmengen für Kinder. Bundesgesundheitsbl - Gesundheitsforsch - Gesundheitsschutz 48, 84–98.

ANNEX 2

Supporting documentation – Residues in honey and other bee's products– selected examples

Amitraz

Amitraz is typically used as a sustained-release strip containing 500 mg of amitraz; however, other modes of application are known as well. The recommended treatment is suspension of two strips per hive for a period of 6 weeks. Other applications involve application of aerosols of emulsions. The metabolism of amitraz yields similar products in animals and plants. The 1998 JMPR evaluation proposes metabolic routes in crops and animals. Metabolism in bees has not been studied; however, the major degradation products occurring in honey are known. These are N-(2,4-dimethylphenyl)-N'-methylformamidine (DMPF) and 2,4-dimethylphenyl-formamide (DMF). DPMF may further degrade to form DMF and 2,4-dimethylaniline (DMA). The marker residue for honey in the EU is the sum of amitraz and all metabolites containing the 2,4-dimethylaniline moiety, expressed as amitraz.

According to European Medicines Agency (EMEA, 1999) a GLP compliant residue depletion study was made available in which six hives that had been treated with the product twice a year, for three successive years, were treated for a period of six weeks. Samples of honey and wax were removed at intervals and analysed using a GC method following conversion of residues to 2,4-dimethylaniline and derivatization. The LOQ was 0.05mg/kg expressed as amitraz for both honey and wax. Residues in honey were stable during storage for up to 4 months at -20°C but were not stable when stored at +25°C. Residues in wax were extremely high. The study cannot be interpreted because the EMEA summary report does not provide sufficient details. Comparably designed studies do not exist.

Table 1: Reported results (EMEA) of a residue depletion study with Amitraz in honey

Days after end of treatment	Honey	Wax
	Mean amitraz equivalents [mg/kg]	
2	0.23	44.7
4	0.10	4.7
15	0.08	72.9

Wallner (1999) has shown that simple contact with beeswax accelerates the degradation of amitraz. An interpretation of these contradictory findings is not possible.

Korta, et al. (2001) characterized the degradation products of amitraz in honey and beeswax. For spiked honey samples a multi-floral commercial honey was used fortified to contain 10 mg/kg; chopped commercial beeswax, previously analyzed to ensure that it was free of amitraz residues was used to prepare fortified wax of 100 mg/kg. The stability in vitro at room temperature was examined over 9 months. In one experiment, the half life of amitraz in honey was 55.2 hours, the half life in bees wax was 6.3 hours. After 15 days, 34 µmoles of amitraz per kg added to honey had degraded to 30 µmoles of DPMF, 27 µmoles of DMF and 3 µmoles of DMA per kg. The range of half lives determined with four different honeys was 12 to 55 hours; however, the molar ratios of DPMF and DMF were nearly constant suggesting a single hydrolysis reaction. Concentrations of DMA were always very low, even at time points of complete breakdown of the parent molecule.

Korta, et al., 2003 has published a GC-MS method for the determination of several acaricides in beeswax. Using this method they analyzed 10 samples of incurred comb wax obtained from hives treated with amitraz in different regions of Spain and France for DMPF the major hydrolysis product of amitraz (recovery of the method 90.9 ± 4.5%). The residue was found in seven samples in concentrations from 0.57 to 33.4 mg/kg. These concentrations correspond to 1.0 to 60 mg/kg of amitraz equivalents.

The above studies seem to contradict each another unless amitraz that is "naturally" incorporated into wax behaves differently from amitraz added to wax by fortification using organic solvents. The issue needs clarification. The basis for the EU MRL is not clear. Beeswax has to be included in MRL considerations. Comb wax is not only used in cosmetics; entire honey combs are offered for sale as gourmet honey. It is eaten by many people with their breakfast sandwich[1].

On the basis of 150g (20g) daily consumption of comb honey (9:1 honey to wax assumed proportions: 135 (18)g honey and 15 (2)g wax), the daily intake on day 2 after treatment according to the EMEA cited GLP study is calculated as: 0.225×0.135 + 44.7×0.015 = 0.7 (0.225×0.018 + 44.7×0.002 = 0.093) mg per person per day. This corresponds to 117 (15.6) % of the JMPR ADI and 389 (51.9) % of the EMEA ADI. 95.7% of this intake results from wax consumption. The bioavailability of amitraz residues from honey and wax is not known.

The daily intake from the use of beeswax as food additive would correspond to 9.6% of the JMPR ADI or 32% of the EMEA ADI on the basis of day 2 residues of the EMEA cited GLP study and the intake estimate made by EFSA. However, use of amitraz as a pesticide can be a significant source of residues in honey and has to be taken into account as well when recommending MRLs.

The EFSA Panel on Food additives, Flavourings, Processing aids and Materials in Contact with Food has reviewed beeswax as a glazing agent and as carrier for flavours (EFSA Journal, 2007). It made a conservative intake estimate of 1290 mg beeswax per person and day. In this assessment honey sold in jars with the honeycomb is also not included.

Tylosin

Tylosin A is converted to Tylosin B (desmycosin) in acidic aqueous solutions (Paesen, et al., 1995; Kochansky, 2006) studied the stability of tylosin in samples of honey containing tylosin residues. Tylosin and desmycosin, were isolated from diluted honey samples by solid-phase extraction followed by high performance liquid chromatography. Tylosin converted to desmycosin exponentially with half-lives of about 102 days at 34°C, 9 days at 50°, 9 hours at 80°, and 48 minutes at 110°. The desmycosin then decayed to unknown products.

The stability of tylosin in sucrose solutions was also investigated (Kochansky, et al., 2006). Stability was tested at (very high) concentrations of 200 mg/L using HPLC with UV detection. When the experiments were carried out in 70% highly purified sucrose at 34 °C tylosin disappeared linearly with time. The half life for tylosin (tylosin A) was approximately 186 days. The authors measured also the concentrations of the other members of the tylosin complex and estimated a half life of approximately 287 days for the entire complex. Inhibition zones were determined using *P. larvae*. The diameter of the zones remained constant over the observation period of 50 days. This underlines that it is not possible to determine tylosin A using microbiological assays since other members of the complex also exhibit significant inhibition of *P. larvae*.

[1] The toxicological monograph on beeswax from the 39th JECFA meeting states: "It is generally believed that waxes are not digested absorbed from the alimentary tract in most mammals, including man. Beeswax may be indigestible in mammals due to the structure of its component compounds, which are not susceptible to hydrolysis by enzymes of the alimentary tract, and due to its insolubility in water and high melting point (62 °C - 65 °C) which prevent dissolution at body temperature. There are no original research data available to support this claim". The toxicological monograph on beeswax from 65th JECFA meeting states: "There is evidence that some solubilisation of beeswax is mediated by the action of bile acids, at least in some species". On dietary exposure the Committee made very conservative assumptions (e.g., a person consumes all foods, etc., containing beeswax at the highest percentile) and arrived at a daily intake of < 650 mg/person. This estimate does not include honey sold in jars with the honeycomb. Honey combs are typically sold in portions of 340 g in Germany.

The US FDA has approved tylosin tartrate for the control of American foulbrood in honey bees. Supporting aggregated residue data are contained in the Freedom of Information (FOI) summary published by the US FDA. The marker residue is parent tylosin. The analytical method for the detection of residues of tylosin in honey used in the residue study is a microbiological assay using an oxytetracycline-resistant strain of *Paenibacillus larvae.* This assay is not selective for the marker residue, parent tylosin. The following details of the residue study are given:

- Test animals: Honey bee, *Apis mellifera*, 40,000 workers/colony
- Treatment groups:
- Untreated controls (4 colonies)
- 200 mg tylosin in 20 g confectioner sugar (1X; 4 colonies)
- 1000 mg tylosin in 20 g confectioner sugar (5X; 4 colonies)
- Duration of treatment: Once every seven days for a total of three treatments (21 days).

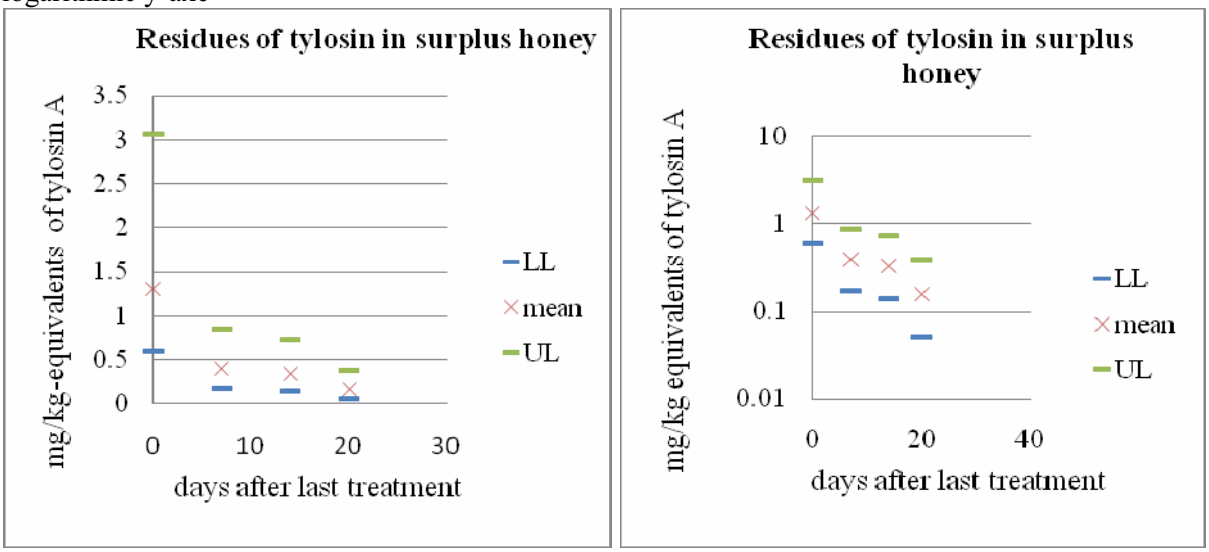
Honey was sampled from the honey supers (surplus honey) between the first and second treatments and from the honey supers and the brood chamber weekly for three weeks following the final treatment. The results are expressed in mg/kg and given for the mean concentrations, lower and upper 95% confidence limits of the mean. The results are summarized in table 2.

Table 2: Residues of Tylosin in honey reported in FOI Summary NADA 013-076

Treatment	Sample from:	0 days			7 days			14 days			21 days		
		Concentration of tylosin residues in mg/kg											
		L95% CL	Mean	U95% CL	L95% CL	Mean	U95% CL	L95% CL	Mean	U95% CL	L95% CL	Mean	U95% CL
200 mg	Brood chamber				0.66	1.45	3.46	0.21	0.47	1.04	0.17	0.40	0.88
1000 mg					2.20	5.55	17.46	1.85	4.52	13.39	0.87	1.98	4.90
200 mg	Surplus	0.59	1.30	3.06	0.17	0.39	0.85	0.14	0.33	0.73	0.05	0.16	0.38
1000 mg		3.21	8.73	34.27	1.50	3.57	9.90	1.07	2.46	6.33	0.72	1.61	3.85

The confidence intervals of the mean are asymmetric (see columns L95%CL, Mean and U95%CL). This observation suggests that the mean could be a mean obtained on a log-scale. As an example, the results obtained with the 200 mg treatment and in surplus honey are plotted on both a linear and a logarithmic scale. Although the plot on the right side is suggestive of a log-normal distribution as basis for the calculations, the confidence intervals still remain asymmetric. Since individual data are not given it is not clear what the data mean and how they have been calculated. The data suggest a half life of approximately 7 to 9 days for both doses.

Figure 1: Residues of tylosin in surplus honey: Left side with linear y-axe; right side with logarithmic y-axe



Valid instrumental methods are now available to measure the concentrations of all components of the tylosin complex simultaneously (Nozal Nalda , et. al., 2006)[2]. Desmycosin (also referred to as tylosin B) has been identified as the primary degradation product of tylosin in honey (Kochansky, 2003).

The degradation of tylosin in honey has been further investigated by Thompson et al., (2007). In their field trials, tylosin was used in single brood chamber colonies containing approximately 30,000 adult honey bees housed in Langstroth deep hive bodies. The study used higher than intended target dosages of tylosin (600 mg). Two types of formulations were used: 300 mg of tylosin tartrate mixed in 20 g of confectioner's sugar and either 300, 900 or 1500 mg tylosin tartrate incorporated into a 100 g pollen patty. Three successive treatments, spaced 7 days apart were performed in September 2004. For residue determination, 15 g samples of newly deposited honey were collected from colonies in July 2005, approximately 1 week after the start of the summer honey flow (collected representatively across several frames of the brood nest and honey super). The amount of honey in the supers was still relatively low at this time. The stability of tylosin A in honey matrices was investigated by spiking a series of replicate honey samples and storing them in the dark at −20 and 20 °C, respectively. Samples were analyzed at 2-week interval for a period of 16 weeks.

Analyses were performed using LC-electrospray MS/MS with roxithromycin as an internal standard. The method was validated using a series of antibiotic-free honey samples of varying physical appearance (i.e. color and moisture content). No appreciable degradation of tylosin A was observed when stored at −20 °C. Over the same period of time, approximately 20% of the tylosin A had degraded to desmycosin when stored at ambient temperature. The following table summarizes the results obtained in the treatment studies. In addition to the values given by the authors in units of µg/kg they are also expressed as µmoles/kg (one micromole of tylosin A is 916.1 µg; one micromole of desmycosin is 771.9 µg). The results show that it is not appropriate to use tylosin as the only residue to define MRLs for two reasons: the contribution of desmycosin may be significant and the ratio of the two molecules may vary as function of time.

[2] This is only an example.

Table 3: Tylosin and Desmycosin in incurred honey samples (supers) of bee colonies, 294 days following the last treatment with tylosin A tartrate expressed as µg-equivalents of tylosin A / kg

Treatment	Replicate	Tylosin	Desmycosin	Tylosin	Desmycosin	Molar ratio	Total residue (*)
		µg/kg		µmoles/kg			µg/kg
Sugar dust, 3x300 mg per colony	1	179	150	0.20	0.19	1.01	357
	2	150	31	0.05	0.04	1.25	83
	3	32	32	0.04	0.04	0.84	70
	4	<5	<5				
Pollen patty, 3x900 mg per colony	1	29	33	0.03	0.04	0.74	68
	2	64	48	0.07	0.06	1.12	121
	3	<5	<5				
	4	<5	<5				
Pollen patty, 3x1500 mg per colony	1	<5	<5				
	2	<5	<5				
	3	23	19	0.03	0.03	1.02	46
	4	6	7	0.01	0.01	0.72	14

The authors, Nozal Nalda, et al., (2006) also analysed incurred multi-floral honey samples from Spain. The colonies were experimentally treated with technical grade tylosin of unknown composition administered to the animals in mixtures of sugars and food preservatives. 15 bee hives were used of which five served as controls, five received a sugar mixture with 200 mg/kg of tylosin, five other received a sugar mixture with 400 mg/kg of tylosin. The treatment was carried out in spring. Honey was collected from brood chamber combs after complete consumption of the feed plus an additional waiting time of 1 month.

No residues were found in the controls. The residue concentrations found in honey from treated bees were not dose related. For table 4 the original data have been re-calculated in µmoles/kg using the following molecular weights: tylosin A: 916.1, tylosin B: 771.9, Tylosin C: 902.1, and tylosin D: 918.1. Tylosin A usually is the major component (approximately 90% but not less than 80%).

Table 4: Residues of the tylosin family in honey obtained from bee colonies treated with tylosin of technical grade expressed as µg-equivalents Tylosin A per kg

Sample	TA	TB	TC	TD	% TA	Total residue(*)
	Concentration [µmoles/kg]					
201	1.59	0.10		0.12	88.0	1660
202	1.33	0.11		0.12	85.4	1430
203	0.78	0.08		0.02	88.8	800
204	1.13	0.02		0.03	95.7	1080
205	5.55	0.45	0.08	0.20	88.5	5740
401	2.01	0.25	0.01	0.09	85.2	2160
402	4.85	0.34	0.02	0.15	90.4	4910
403	0.65	0.08		0.01	88.1	670
404	2.73	0.36	0.02	0.10	85.1	2940
405	7.42	0.76	0.09	0.23	87.3	7790

Adams, et al., (2007) have studied the depletion of tylosin residues in honey from treated bee colonies. Hives (brood box) were dosed with 1.2 g of tylosin tartrate in 200-250 ml 50-60% sucrose in water (single dose) and honey was analyzed at several intervals over a 20-week period. The dosing

study was carried out from June 2005 to February 2006. During winter colonies were fed with 50% sucrose. Six colonies were treated. Baseline residue levels were determined in up to 100 g of nectar/honey before the treatment in June. Seven days after dosing and honey sampling two randomly selected colonies were shook-swarmed. Each sampling day, four comb samples (approximately 8x10 cm) were taken from each hive, two from brood chamber and two from super. The four samples were taken from different frames. On day 28 each individual sample was analyzed; on the other days brood chamber and super samples were bulked separately before analysis. The analytical method was LC-MS/MS. For the following description of the results all concentration values given by the authors were re-calculated as µmoles/kg**.**

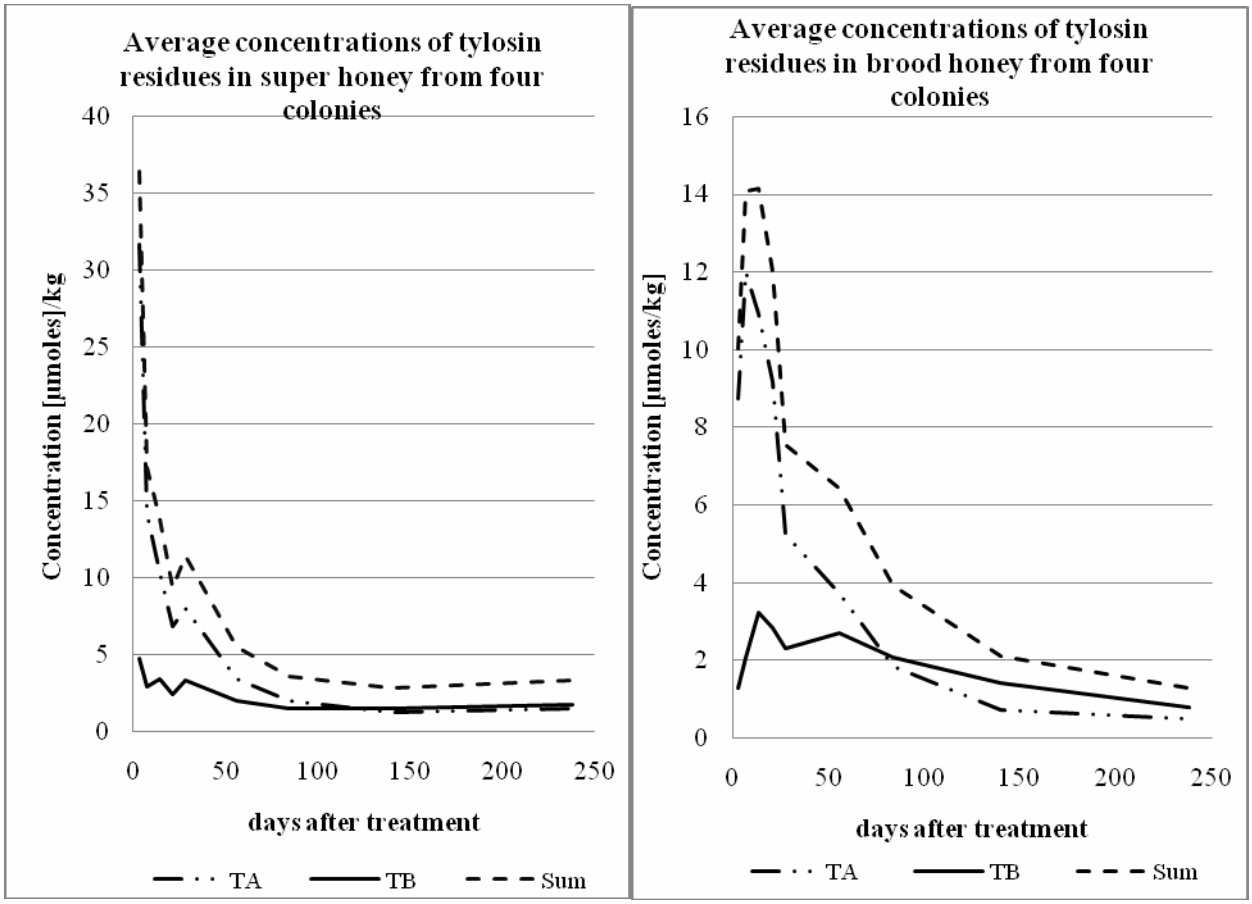
For day 28 the authors provide individual tylosin A data for two samples per hive and type of comb taken from two different frames of either brood comb or super comb. The results are shown in table 5. Although it is immediately evident from the data that these results are not normally distributed the authors calculate mean and standard deviations for all individual eight data for a given time point. The table clearly shows that the variability within a frame can be very high (e.g., nine fold range for colony 1 and brood honey).

Table 5: **Variability of tylosin concentrations within a frame**

Colony	Type of data	Brood honey		Super honey	
		Top	Bottom	Inner	Outer
		Concentration [µmoles/kg]			
1	Individual concentration [µmoles/kg]	5.7	0.7	7.1	6.9
2		3.3	0.8	3.6	3.1
3		11.9	12.8	17.6	20.6
4		4.8	2.3	5.4	12.8
All colonies	n	8		8	
	mean	5.3		9.7	
	s.d.	4.7		6.6	

Using the average values two graphs were prepared showing the kinetic behaviour of tylosin A and tylosin B (desmycosin) in surplus honey and in brood honey. See figure 2.

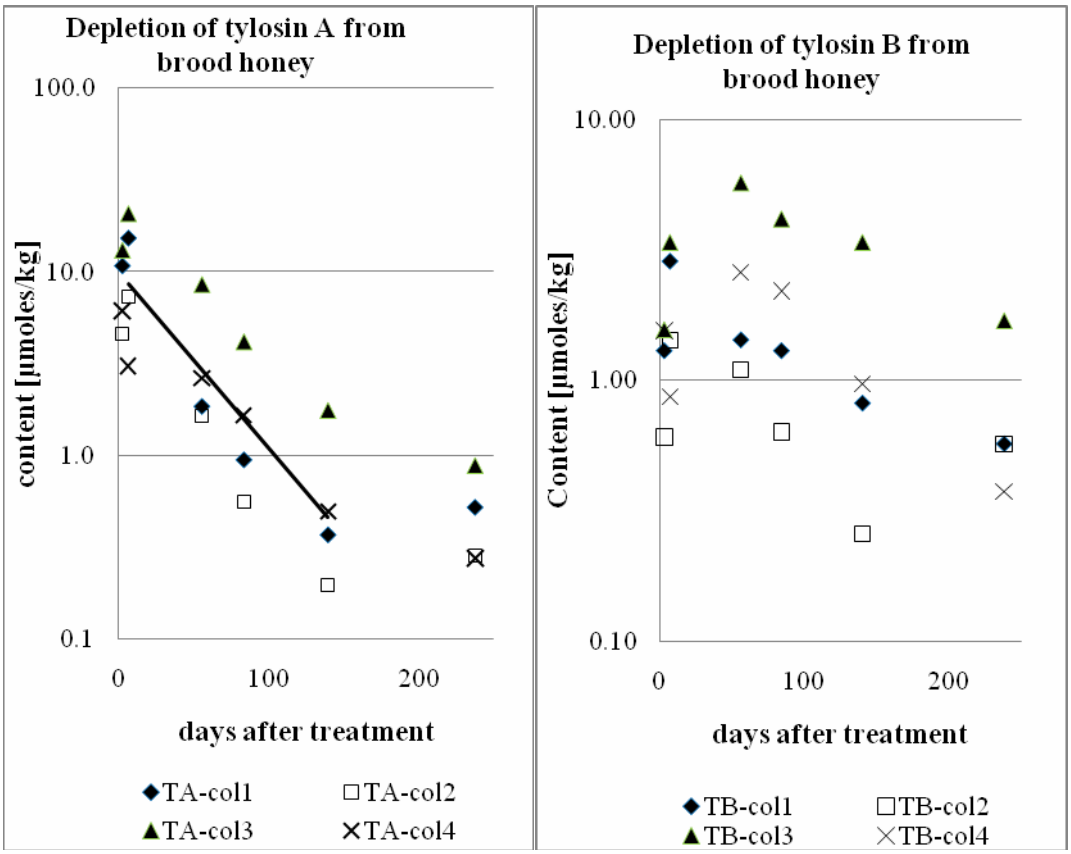
Figure 2: Kinetics of tylosin residues in surplus honey and in brood honey



In surplus honey both tylosin A and desmycosin reach their highest concentrations shortly after the treatment. The concentrations of both compounds decrease with time; tylosin disappears more rapidly. Therefore, at the later time points desmycosin concentrations seem to be slightly higher. In brood honey tylosin A reaches its maximum concentration between 7 and 14 days after treatment and desmycosin concentrations continue increasing until after 14 days. After 84 days concentrations of desmycosin are always significantly higher compared to tylosin A.

For brood honey and for six time points the authors have provided averages for individual colonies. This permits estimates of variability between the colonies. The following figures below (figure 3and 4) show the kinetics of tylosin A and desmycosin in four individual hives.

Figure 3: Depletion of tylosin A and B from brood honey



The two graphs show the great variability in the behaviour of different hives of the same apiary. The depletion curves of tylosin A suggest a log-linear part from 7 to 140 days following treatment. Linear regression analysis using the natural logarithms of the tylosin A concentrations yields the following parameters (n=16):

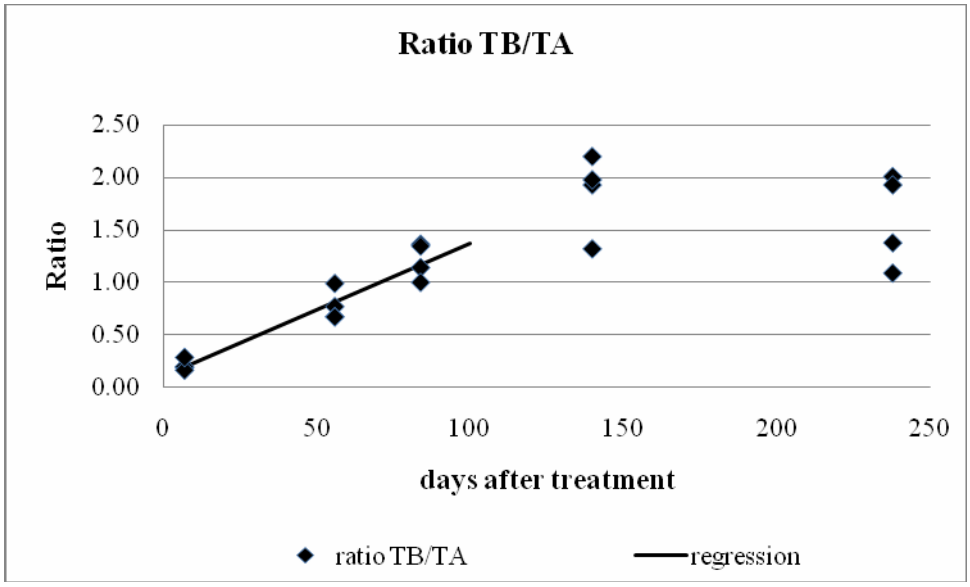
a	2.30294
b	-0.02200
r	-0.81989
$s_{y.x}$	0.78977

From these parameters a depletion half life of 31.5 days for tylosin A in brood honey (for the time period of 7 to 140 days after treatment) can be calculated. The ratio of the concentrations of desmycosin and tylosin A seem to reach a plateau of approximately 1.5; however, for the first approximately 100 days it can also be described by a straight line with time after treatment as the independent variable. The regression parameters using the data points from day 7 to 140 (n=16; unweighted linear regression) are:

a	0.11316
b	0.01253
r	0.95136
$s_{y.x}$	0.20865

The individual data points and the regression line are shown in the next graph (figure 4). Although the number of data points is very limited it seems that the variance of the ratio is not constant over time and weighted regression would be more appropriate.

Figure 4: Ratio of the concentrations of desmycosin and tylosin A in brood honey



For the concentrations of tylosin A and desmycosin in surplus honey no individual data were provided; however, from the average values given for days 7 to 140 after treatment an estimated half life is 40 days.

The EMEA ADI for tylosin is 0.36 mg/kg bw/day or 21.6 mg/day for a 60 kg person. In the following table the sum of the average concentrations of tylosin A and desmycosin expressed as tylosin A has been calculated and the estimated daily intake of a 60 kg person consuming 150 (20) g of honey per day has been calculated in percent of the ADI (under the conditions of the Adams study; data on residues in wax were not included in the study).

Table 6: Theoretical consumption of "total residues" of tylosin by a 60 kg person using the conditions in the Adams et al. (2007) study

Withdrawal time [days]	% EMEA ADI equivalents consumed with 150 (20) g of Honey
3	23.2 (3.1)
7	10.9 (1.4)
14	8.8 (1.2)
21	5.9 (0.8)
28	7.3 (1.0)

The table 7 summarizes some half life estimates for tylosin A obtained in various studies, either *in vitro* (degradation) or *in vivo* (depletion). *In vivo* the actual concentration found is a complex function of uptake, degradation, metabolism and dilution. Unfortunately none of the studies cited above provide any information on the time course of the amounts of honey in the hives, supers, frames, etc. Therefore, the effect of dilution cannot be estimated. A general conclusion is the half life of tylosin A *in vivo* is much shorter than *in vitro*. The contribution of the individual factors mentioned above is not known.

Table 7: "Half life" of tylosin A *in vitro* and *in vivo*

Authors	Type of study	Initial concentration	Temperature [°C]	Half life [days]
Kochansky, J. (2006). Journal of Apicultural Research and Bee World, 45(2), 32-36.	In vitro in honey	Not given	34	102
Kochansky J., Knox D., Shimanuki H. (1999). Apidologie, 30, 321-326.	In vitro in sucrose	200 mg/L in 70% sucrose	34	186
Freedom of Information Summary, Supplemental New Animal Drug Application, NADA 013-076.	In vivo depletion in surplus honey	0.2-8.7 mg/kg of microbiologically active residues	Not given	7-9 (days 0-21)
Thompson, T. S., Pernal, S. F., Noot, D. K., Melathopoulos, A. P., van den Heever, J. P. (2007). Analytica Chimica Acta, 586 (1-2), 304-311.	In vitro in honey	Not given	Ambient	20% decay in 16 weeks
Adams, S.J., Heinrich, K., Hedmanski, M., Fussell R.J., Wilkins, W., Thompson, H.M., Sharman, M. (2007). Apidologie, 38, 315-322.	In vivo depletion in brood honey	2.8-19 mg/kg on day 7 after treatment	Not given	31.5 (days 7-140)
	In vivo depletion in surplus honey	13 mg/kg average for four hives on day 7		About 40 (days 7-140)

It has been known for long time that many antibiotics are degraded in honey. For example, microbiological inhibition assays were used to investigate the stability of certain antibiotics in sterile honey and in sugar syrup at both 34 and 4 °C. The following table is extracted from an old original publication Landerkin and Katznelson (1956). Microbiological inhibition tests are generally unable to quantify individual compounds in mixtures, but qualitative trends can be established.

Table 8: Stability of some antibiotics in honey

Month:	Start	1	2	3	4	5	6	7	8	9
	Concentration [mg/kg] in honey at 4 °C									
Erythromycin	110	100	90	90	70	70	68	85	80	60
Tetracycline	110	50	40	40	35	37	36	48	54	24
Oxytetracycline	108	75	32	22	24	27	28	38	17	15
Chlorotetracycline	84	80	27	32	38	45	42	32	40	36
Streptomycin	80	65	57	50	37	32	30	42	31	35
	Concentration [mg/kg] in honey at 34 °C									
Erythromycin	115	45								
Tetracycline	115	45	35	35	18	13	12	15	5	2
Oxytetracycline	90	20								
Chlorotetracycline	88	70	23	16	14	18	16	5	3	2
Streptomycin	80	60	54	20	20	21	12	31	18	10

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AVILAMYCIN

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Richard Ellis, South Carolina, United States

IDENTITY

1.1 International non-proprietary name (INN): Avilamycin

1.2 Synonyms and abbreviations: CGA 59 327 and EL-750.

1.3 International Union of Pure and Applied Chemistry (IUPAC) name:

Avilamycin factor A:
O-(1R)-4-C-acetyl-6-deoxy-2,3-O-methylene-D-galactopyranosylidene-(1'3-4)-2-O-(2-methyl-1-oxopropyl)-α-L-lyxopyranosyl O-2,6-dideoxy-4-O-(3,5-dichloro-4-hydroxy-2-methoxy-6-methylbenzoyl)-β-D-arabino-hexopyranosyl-(1'4)-O-2,6-dideoxy-D-arabinohexopyranosylidene-(1'3-4)-O-2,6-dideoxy-3-C-methyl-β-D-arabinohexopyranosyl-(1'3)-O-6-deoxy-4-O-methyl-β-D-galactopyranosyl-(1'4)-2,6-di-O-methyl-β-D-mannopyranoside

Avilamycin factor B:
O-4-C-acetyl-6-deoxy-2,3-O-methylenehexo-pyranosylidene-(1'3-4)-2-O-acetyl-L-lyxopyranosyl O-2,6-dideoxy-4-O-(3,5-dichloro-4-hydroxy-2-methoxy-6-methylbenzoyl)-β-D-arabino-hexopyranosyl-(1'4)-O-2,6-dideoxy-D-ribo-hexopyranosylidene-(1'3-4)-O-2,6-dideoxy-3-C-methyl-D-arabino-hexo-pyranosyl-(1'3)-O-6-deoxy-4-O-methyl-β-D-galactopyranosyl-(1'4)-2,6-di-O-methyl-D-mannopyranoside.

1.4 Chemical Abstract Service number:

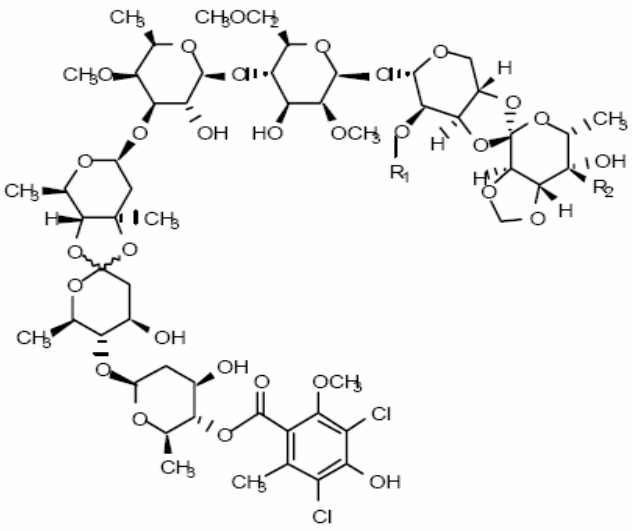
Avilamycin A: 69787-79-7; Avilamycin B: 73240-30-9

1.5 Structural formula: See next page

1.6 Molecular Formula: Avilamycin A: $C_{6l}H_{88}Cl_2O_{32}$ Avilamycin B: $C_{59}H_{84}Cl_2O_{32}$

1.7 Molecular Weight: Avilamycin A: 1403; Avilamycin B: 1375

Figure 1: Structural formula of main avilamycin components



Avilamycin A: $R_1 = COCH(CH_3)_2$, $R_2 = COCH_3$

Avilamycin B: $R_1 = COCH_3$, $R_2 = COCH_3$

Melting point:

Avilamycin A: 166-169°C
Avilamycin B: 179-182°C

OTHER INFORMATION ON IDENTITY AND PROPERTIES

Pure active ingredient:

Avilamycin is an orthosomycin antibiotic complex produced by the fermentation of Streptomyces viridochromogenes. Orthosomycin antibiotics are divided into two groups: those that contain an aminocyclitol residue and those that are esters of dichlorisoeverninic acid. Avilamycin is in the latter group as are the evernimicins. Avilamycin complies with the following specifications for the composition of the total factor content.

Avilamycin A:	Not less than 60%
Avilamycin B:	Not more than 18%
Avilamycin A + Avilamycin B:	Not less than 70%
Other single Avilamycin factors:	Not more than 6%

Typical Avilamycin content is 260 mg activity/g.

Sixteen minor factors have been specifically identified. Their molecular and structural formulas are given below.

Structures of Avilamycin Factors

Factor	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	Molecular Formula	Molecular Weight
A	$-CO-CH(CH_3)_2$	$-CO-CH_3$	$-OCH_3$	-Cl	-Cl	$-CH_3$	$-CH_3$	$-OCH_3$	$C_{61}H_{88}Cl_2O_{32}$	1403
A'	$-CO-CH_2CH_3$	-H	$-OCH_3$	-Cl	-Cl	$-CH_3$	$-CH_3$	$-OCH_3$	$C_{58}H_{84}Cl_2O_{31}$	1347
B	$-CO-CH_3$	$-CO-CH_3$	$-OCH_3$	-Cl	-Cl	$-CH_3$	$-CH_3$	$-OCH_3$	$C_{59}H_{84}Cl_2O_{32}$	1375
C	$-CO-CH(CH_3)_2$	$-CHOH-CH_3$	$-OCH_3$	-Cl	-Cl	$-CH_3$	$-CH_3$	$-OCH_3$	$C_{61}H_{90}Cl_2O_{32}$	1405
D_1	-H	$-CO-CH_3$	$-OCH_3$	-Cl	-Cl	$-CH_3$	$-CH_3$	$-OCH_3$	$C_{57}H_{82}Cl_2O_{31}$	1333
D_2	$-CO-CH_3$	$-CHOH-CH_3$	$-OCH_3$	-Cl	-Cl	$-CH_3$	$-CH_3$	$-OCH_3$	$C_{59}H_{86}Cl_2O_{32}$	1377
E	-H	$-CHOH-CH_3$	$-OCH_3$	-Cl	-Cl	$-CH_3$	$-CH_3$	$-OCH_3$	$C_{57}H_{84}Cl_2O_{31}$	1335
F	$-CO-CH(CH_3)_2$	$-CO-CH_3$	-OH	-H	-Cl	$-CH_3$	$-CH_3$	$-OCH_3$	$C_{60}H_{87}ClO_{32}$	1355
G	$-CO-C_4H_9$	$-CO-CH_3$	$-OCH_3$	-Cl	-Cl	$-CH_3$	$-CH_3$	$-OCH_3$	$C_{62}H_{90}Cl_2O_{32}$	1417
H	$-CO-CH(CH_3)_2$	$-CO-CH_3$	$-OCH_3$	-Cl	-H	$-CH_3$	$-CH_3$	$-OCH_3$	$C_{61}H_{89}ClO_{32}$	1369
I	$-CO-CH_2CH_3$	$-CO-CH_3$	$-OCH_3$	-Cl	-Cl	$-CH_3$	$-CH_3$	$-OCH_3$	$C_{60}H_{86}Cl_2O_{32}$	1389
J	$-CO-CH(CH_3)_2$	$-CO-CH_3$	$-OCH_3$	-Cl	-Cl	-H	$-CH_3$	$-OCH_3$	$C_{60}H_{86}Cl_2O_{32}$	1389
K	$-CO-CH(CH_3)_2$	$-CO-CH_3$	$-OCH_3$	-Cl	-Cl	$-CH_3$	$-CH_2OH$	$-OCH_3$	$C_{61}H_{88}Cl_2O_{33}$	1419
L	$-CO-CH(CH_3)_2$	-CO-H	$-OCH_3$	-Cl	-Cl	$-CH_3$	$-CH_3$	$-OCH_3$	$C_{60}H_{86}Cl_2O_{32}$	1389
M	$-CO-CH(CH_3)_2$	$-CO-CH_3$	$-OCH_3$	-Cl	-Cl	$-CH_3$	-H	$-OCH_3$	$C_{60}H_{86}Cl_2O_{32}$	1389
N	$-CO-CH(CH_3)_2$	$-CO-CH_3$	$-OCH_3$	-Cl	-Cl	$-CH_3$	$-CH_3$	-OH	$C_{60}H_{86}Cl_2O_{32}$	1389

Solubility:

Solubility in water and organic solvents is expressed in g/L. The solubility of avilamycin factor A has been determined in a variety of solvents at 20°C.

Solvent	Solubility (g/L)
Water	1
Ethanol	4
Methanol	5
Ethyl acetate	10
Acetone	50
Heptane	< 1
Chloroform	100

Refractive index, optical rotation:

The optical rotation of a 2.773% solution of factor A in dioxane was $\alpha_D^{20} = + 2° \pm 1°$.

RESIDUES IN FOOD AND THEIR EVALUATION

The residue studies were carried out using avilamycin as a fermentation product, with different degrees of purity or as pure (crystalline) product. The factor composition and purity of avilamycin differed between studies.

The Committee evaluated avilamycin to recommend MRLs in poultry, pigs and rabbits at the request of the 17th session of the Codex Committee on Residues of Veterinary Drugs in Foods.

Conditions of use

Avilamycin is intended for use as a veterinary medicine in chickens, turkeys, pigs and rabbits to control bacterial enteric infections. It exhibits good antimicrobial activity against important veterinary Gram-positive pathogens (e.g., *Clostridium perfringens*) and has no related molecules in its class in human use. Therefore, avilamycin has been developed for treating necrotic enteritis in poultry, and enteric disease in pig and rabbits.

Avilamycin was previously authorised in the European Union (EU) as a feed additive for growth promotion in accordance with Council Directive 70/524/EEC; the substance was incorporated in pig feedstuffs at a concentration of 20 mg/kg feed for animals up to 6 months of age and 40 mg/kg feed for animals up to 4 months of age. It was incorporated into chicken and turkey feedstuffs at a concentration of 10 mg/kg feed. The use of the substance as a feed additive was discontinued in the EU from 1 January 2006.

Dosage

Table 1: Recommended doses and duration of treatment for Avilamycin in feed

Target Animal	Dose in Feed (mg/kg)	Dose Rate (mg/kg bw/day)	Maximum Duration (days)
Pig	100	6-8	21 days
Chicken	100	20	21 days
Turkey	100	20	21 days
Rabbit	80	5	28 days

PHARMACOKINETICS AND METABOLISM

Pharmacokinetics in Laboratory Animals, Humans and Food Animals

No classical pharmacokinetic studies have been conducted in any species with avilamycin because avilamycin is not detectable in plasma (LOD = 0.05 mg/kg) following oral administration of avilamycin in feed. In addition, the concentration necessary for kinetic analysis would be well below the toxicologically relevant concentrations and would not be pertinent to human food safety. Metabolism and Residue studies in pigs, poultry and other species (rat) that have been conducted using radiolabelled material are presented below.

Where blood, serum or plasma concentrations were measured in various species following oral doses, avilamycin concentrations were below the limits of detection. For example, in broiler chickens that were fed with a ration containing 22 mg of avilamycin/kg of feed for 25 days, no avilamycin was detected in blood measured by a bio-autographic method (LOD <0.04 mg/kg) or by GC method (LOD <0.1 mg/kg) (West, et al., 1982).

Humans

Avilamycin has not been developed for human use and therefore, no pharmacokinetic data in humans are available.

Laboratory animals

Avilamycin is primarily excreted in faeces when administered orally to pig or chickens. In a GLP compliant rat study (Magnussen, 1985a), less than one percent of the oral dose was eliminated in the urine after 72 hours, while 80 to 104% was recovered in the faeces.

Pigs

In a balance-excretion non GLP-compliant study two cross-bred gilts were administered non-radiolabelled avilamycin at 120 mg per kg in the feed per day (Dalidowicz, et al., 1983). After 7 days administration to approximate steady state conditions, a single bolus dose of 120 mg of [U-^{14}C]avilamycin was administered and excreta were collected at 24-hr intervals for 9 days. During the collection period, the two gilts excreted 96.9% and 99.0% of the dose, respectively, with an average of 93.4% in the faeces and 4.5% in the urine. The bulk of the radioactivity was excreted within the first four days. Another radiolabelled GLP-compliant pig study (Magnussen, et al., 1987) conducted on six crossbred pigs receiving the same dose for either ten or fourteen days showed similar results. Excreted radioactivity reached a plateau after 2-3 days and, on average comprised 8% in urine and 92% in faeces. Approximate concentration in faeces was 120 mg/kg equivalents avilamycin. Results are shown in Table 2.

Table 2 : Excreted radioactivity in pigs fed ten days with avilamycin

Collection Period (day)	Urine (µCi)	Faeces (µCi)
1	0.76	1.12
2	1.06	18.56
3	1.45	17.57
4	1.51	18.83
5	1.53	16.96
6	1.54	18.83
7	2.04	21.09
8	1.69	21.26
9	1.94	20.29
10	1.57	18.16

Chickens

A balance-excretion non-GLP-compliant study was conducted in chickens (Dalidowicz, et al., 1984a). Broiler chickens (2 males/2 females) were administered non-radiolabelled avilamycin at 20 mg of microbiological activity per kg in the feed. After 7 days administration to approximate steady state conditions, a single bolus dose of 4 mg of [U-^{14}C]avilamycin was administered and excreta were collected at 24-hr intervals for 13 days. During the collection period, the birds excreted 92.8%, 99.2%, 96.6% and 84.4% of the dose, respectively. An average of 90% of the radioactivity was excreted within the first 6 days.

Data for avilamycin in turkeys and rabbits are not available.

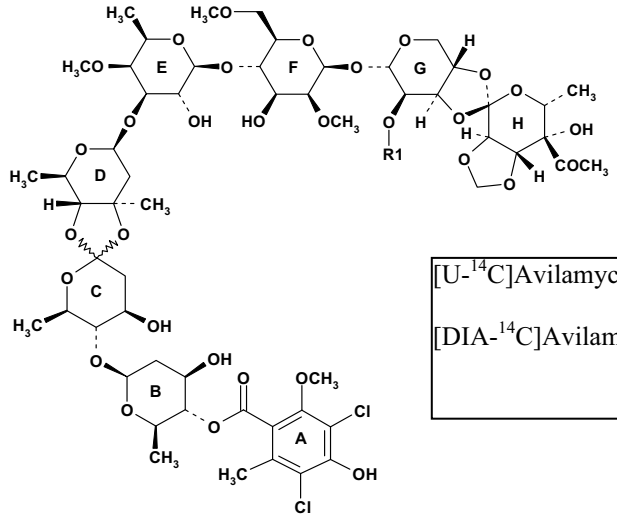
Metabolism in Laboratory Animals

Position of radiolabel (^{14}C) in Avilamycin.

The pivotal residue and metabolism studies for pig and chickens were performed using radiolabelled avilamycin. However, because avilamycin is extensively metabolized, the position of the radiolabel is important in understanding not only the metabolic profile of this substance, but also the correct interpretation of the total radioactive tissue data. Therefore, a discussion of the radiolabel position is necessary prior to the assessment of the specific studies.

Two types of radiolabelled avilamycin have been prepared by fermentation using *S. viridochromogenes* with one of two radiolabelled precursors:

Figure 2: Structure of radiolabelled avilamycin



[U-^{14}C]Avilamycin:
All rings labelled
[DIA-^{14}C]Avilamycin
85% Ring A
15% other (possibly R1)

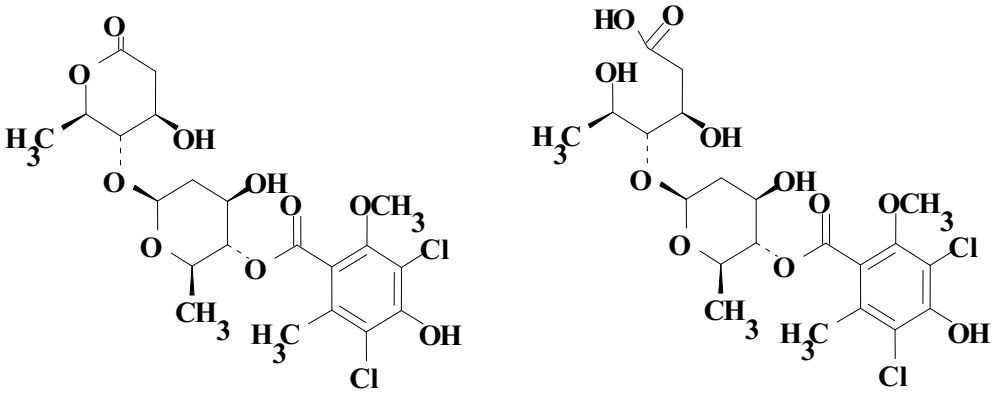
1. [U-^{14}C]avilamycin: Using uniformly labelled glucose, [U-^{14}C-glucose], as the precursor labels the molecule uniformly in all rings (Donoho et al, 1987).
2. [DIA-^{14}C]avilamycin: Using [2-^{14}C-diethylmalonate] as the precursor places approximately 85% of the radioactivity in the dichloro-isoeverninic acid moiety (DIA; Ring A in Figure 2). The remaining 15% of the radioactivity was not conclusively identified, but is suggested to be associated with the iso-butyrate, propionate or acetate moiety at position R1 on Ring G (Dalidowicz, 1985).

Rats

In a GLP- compliant study (Magnussen, 1985a), three male and three female Sprague-Dawley rats weighing 215-265 grams each were dosed by gavage for three consecutive days with [DIA^{14}C]avilamycin (specific activity 0.246 µgCi/mg) at levels equivalent to 100 mg/kg of body weight. Following the initial dose, urine and faeces were collected separately from each animal at 24-hour intervals.. In addition, faeces collected during the 24-hour period following the third dose were extracted and assayed for avilamycin and metabolites. All radioactivity in the selected faeces samples was extractable into ethyl acetate at a neutral and acidic pH.

The neutral fraction contained 85-87% of the radioactivity, while the acidic fraction contained 12-14%. TLC analysis showed avilamycins A and B to represent 40-60% of the radioactivity in the neutral fraction, while an unidentified, polar metabolite represented 10-30%. The major radioactive component in the acidic fraction was confirmed as flambalactone (previously identified as the major avilamycin derived residue in pig liver (Magnussen, 1985b). It is formed by cleavage of the ortho ester linking the C and D rings of avilamycin. Flambalactone represented 30-60% of the radioactivity in the acidic fraction. One other metabolite common to both rats and pigs in the acidic fraction representing 10-30% of the radioactivity was later identified as flambic acid (Magnussen, et al., 1987). In other studies flambalactone and flambic acid were found to be inter-convertible.

Figure 3: Structures of Flambic acid and Flambalactone



Metabolism in Food Producing Animals

Pigs

Avilamycin is extensively metabolized and exhibits low tissue residues when administered orally to pigs (Magnussen, et al., 1991). This publication summarizes the findings of multiple pig and rat metabolism studies (Dalidowicz, 1985; Dalidowicz, et al., 1983; Magnussen, et al., 1984; Magnussen & Herberg, 1985; Magnussen, et al., 1987; Magnussen, 1985a; Magnussen, 1985b; Donoho & Magnussen, 1987). These studies comprise the pivotal metabolism work in pigs.

Regardless of the radiolabel position, parent avilamycin in pig liver was not observed above the limit of quantitation. Following administration of [DIA-^{14}C]avilamycin to pigs in the feed, parent avilamycin was reported as 'not detected' in the liver (LOD = ca. 0.01 mg/kg) Magnussen, 1985b). Following administration of [U-^{14}C]avilamycin to pigs in the feed (GLP-compliant study), the authors reported radioactivity in the silica gel chromatography fraction where avilamycin was expected to elute (Magnussen, et al.,1987). This fraction accounted for less than 10% of the radioactive residue in liver (<0.02 mg/kg).

In pig faeces, less than about 5% of the total radioactivity was identified as parent avilamycin (Magnussen, 1985b). Avilamycin A constituted approximately 8% of the total faecal residue in pigs and 19% of the faecal radioactivity of the rat [U^{14}C- avilamycin] (GLP-compliant study), (Donoho and Magnussen, 1987). No avilamycin was detected in pig urine following administration of [DIA-^{14}C]avilamycin in the feed (Magnussen, 1985b). Only one major metabolite was identified in pig and rat samples -flambic acid. During the initial metabolite characterization it was thought that flambalactone was the major metabolite, comprising 45 to 50% of the faecal and urine radioactivity and 15 to 20% of liver residue (Magnussen, 1985a). Flambalactone was proposed as an artefact of the isolation of flambic acid from pig liver (Magnussen, 1985b), and this conclusion was substantiated in the study conducted by Magnussen et al., 1987.

In subsequent studies, the authors considered the hypothesis that flambic acid was most likely formed *in vivo*, given that the conversion of flambic acid to flambalactone occurs in organic solvent. In this latter study, flambic acid was the major metabolite in the faeces, but quantitation was not reported. A subsequent publication stated that flambic acid constituted 6 to 8% of the total liver radioactivity (Magnussen, et al., 1991). The actual liver concentration of flambic acid in the two studies appeared to be similar, but the total radioactivity was higher in the study conducted by Magnussen, et al., 1987 where [U-^{14}C]avilamycin was used. This resulted in a lower percent of total radioactivity for this metabolite (Magnussen, et al., 1991).

No other significant metabolites were identified in pigs or rats, although a few minor peaks were observed. The silica gel chromatographic profiles of extracts from the faeces, urine and livers of rats and pigs treated with [U-^{14}C]avilamycin were qualitatively comparable and quantitatively similar by visual inspection (Donoho & Magnussen, 1987). There was a good correlation between the metabolic profiles of rats and pigs.

The faeces extract from [DIA-^{14}C]avilamycin-treated pigs exhibited the same three peaks, but the proportions were different, with the flambic acid-containing peak predominant. Additional TLC analyses of the column fractions from faeces extracts indicated that oligosaccharide-derived metabolites were present in [U-^{14}C]avilamycin samples that were not present in [DIA-^{14}C]avilamycin samples. These metabolites were not further characterized because the corresponding peaks were not present in liver and would thus not pose a food safety risk.

The metabolic profiles in liver of treated rats and pigs were essentially the same with the flambic acid as the most abundant metabolite (Donoho & Magnussen, 1987). Parent avilamycin concentration in rat and pig liver were less that 0.05 mg/kg. The pattern of minor metabolites was similar but insufficient for identification. Data are supportive that rats treated with avilamycin have been exposed to the same metabolites that are present in edible tissues of treated pigs.

Characterization of residues in fat samples from treated pigs demonstrated that essentially all of the residues in fat are due to the incorporation of radioactivity into the endogenous fatty acids, oleic and stearic acid (Dalidowicz, 1985). No DIA-related residues were detected in fat when assayed by hydrolysis and GC analysis, indicating that parent and DIA-containing metabolites such as flambic acid are not detectable (Magnussen, et al., 1984). Moreover, when the radiolabel is distributed into the carbohydrate moieties of avilamycin (i.e., [U-^{14}C]avilamycin), the total radioactive residues are higher than those when using [DIA-^{14}C]avilamycin, while the amounts of DIA-containing residues remain relatively constant (Magnussen, et al., 1984;, Magnussen, et al.; 1987, Magnussen, et al., 1991) The increased incorporation of carbon-14 into fatty acids when [U-^{14}C]avilamycin was administered is consistent with the avilamycin carbohydrate moieties being extensively metabolized.

Chickens, Turkeys and Rabbits

No metabolism data available.

TISSUE RESIDUE DEPLETION STUDIES

Radiolabelled Residue Depletion Studies

Pigs

Several GLP-compliant studies following administration of ^{14}C avilamycin were submitted. Two of the studies used [DIA^{14}C] avilamycin and the third used [U-^{14}C] avilamycin.

Five crossbred pigs, three gilts and two barrows, weighing approximately 46 kg each were fed at 12-hour intervals for seven days with a ration containing 76mg of [DIA^{14}C] avilamycin per kilogram of feed (equivalent to 80 mg/kg of activity and equal to 4.6-6.1 mg avilamycin/kg bw/day) (Magnussen & Herberg, 1985). Each day, animals received an amount of ration equal to 4% of their body weights. At a practical zero-time withdrawal (six hours) after the final medicated feed ration, one gilt was sacrificed. The remaining animals were then fed non-medicated ration at 12-hour intervals for either three or five days, and one gilt and one barrow sacrificed at the end of each time period. At each sampling time, muscle, liver, kidney, and fat were collected for radiochemical analysis. Results are shown in Table 3.

Table 3: Total radiolabel residue (TRR) in pig tissues

Withdrawal		TRR (mg/kg equivalents avilamycin)			
Days	n	Muscle	Liver	Kidney	Fat
0	1	NDR[1]	0.15	0.08	0.07
3	2	NDR	NDR	0.02	0.05
5	2	NDR	NDR	< 0.03[2]	0.05

[1:] NDR = no detectable residue
[2:] one animal, 0.025 mg/kg; one animal NDR (0.017 mg/kg)

At zero-time withdrawal, no detectable residue was found in muscle, while the total radiolabel residues in liver, kidney, and fat, expressed as avilamycin equivalents, were 0.15, 0.08, and 0.07 mg/kg, respectively. After a three-day withdrawal period, no residues were detected in either liver or muscle, while residues in kidney and fat were 0.024 and 0.053 mg/kg, respectively. After five days, residue levels in fat were nearly the same as those observed at three days, while levels in kidney were from non-detectable residues to 0.025 mg/kg.

Concentrations of avilamycin-related radioactivity in liver and muscle declined to non-detectable levels within three days after the termination of dosing, while concentrations in kidney declined to near non-detectable levels within five days after the termination of dosing. Radioactivity in fat showed a much slower rate of decline due to the fact that radiolabelled carbon from the ^{14}C-avilamycin molecule had become incorporated into the fatty acid fraction as demonstrated by Dalidowicz, 1985. Authors quoted a fat turnover rate of 14-21 days but provided no evidence to support the comment.

In another study conducted by Magnussen, et al, 1984, nine crossbred pigs, weighing approximately 44 kg each, were fed with a ration containing 76.2 mg of [DIA^{14}C]avilamycin per kilogram of feed (equivalent to 80 mg/kg of avilamycin activity and equal to 4.6-6.1 mg avilamycin/kg bw/day) at 12-hour intervals for either four, seven, or ten days. Each day, animals received a ration equal to 4.0% of their body weights, equivalent to a daily dose of approximately 134 mg of DIA^{14}C-avilamycin. All animals were sacrificed at a practical zero-time withdrawal (six hours) after the final feeding. Muscle, liver, kidney, fat, and bile were collected for radiochemical analyses by liquid scintillation counting. Selected tissues were assayed for avilamycin by bio-autography and residues containing the dichloroisoeverninic acid (DIA) moiety. Liver from each animal was extracted to determine levels of non-extractable radioactivity. For total radioactive residues (TTR) results are shown in Table 4.

Table 4: Total radiolabel residue (TTR) in pig tissues.

Dosing	Total Radioactivity (mg/kg equivalents avilamycin)				
Interval (days)	Muscle	Liver	Kidney	Fat	Bile
4	0.01	0.21	0.10	0.05	18.9
7	0.01	0.23	0.10	0.08	19.9
10	0.02	0.22	0.10	0.12	19.8

After ten days dosing, total mean radiolabel residues in liver, fat, and kidney, expressed as avilamycin equivalents, were 0.22, 0.12, and 0.10 mg/kg, respectively. Residues in muscle were less than 0.016 mg/kg. Steady-state concentrations of radioactivity were attained in muscle, liver, and kidney within four days after the initiation of dosing. A steady-state concentration was not attained in fat during this study. The study mentioned in the metabolism section conducted by Dalidowicz, 1985 demonstrated that radioactivity found in fat was incorporated into the fatty acid portion of triglycerides. These non-active residues were not of toxicological concern.

Liver, kidney, and fat from animals dosed for ten days were assayed for avilamycin by bio-autography (Prichard et al, 2006; Method Number AM-AA-CA-R075-AB-755). This method consisted of extracting avilamycin from pig or broiler tissues with acetone. The acetone extract is purified by liquid-liquid partitioning, and the purified extract is spotted on a thin layer chromatographic plate (TLC). After development the TLC plate is subjected to bio-autographic analysis using a *Micrococcus flavus* overlay. The plate is sprayed to enhance the appearance of the zones of inhibition and the presence or absence of avilamycin is determined by comparison with a reference standard. The method does not determine the concentration of avilamycin, but the LOD was reported at 0.05 mg/kg. Results are presented in Table 5.

Table 5: Microbiologically active avilamycin pig tissue residues

Dosing Interval (days)	Microbiological Activity (mg/kg equivalents avilamycin)			
	Muscle	Liver	Kidney	Fat
10	--	< 0.05	NDR[1]	NDR[1]

NDR[1]: non-detectable residues.

No microbiologically active residues of avilamycin were detected in kidney or fat and only traces in liver, but were considerably less than the limit of detection (LOD is < 0.05 mg/kg). Muscle was not assayed due to radioactivity concentrations less than LOD for the bio-autographic assay. Tissue residues containing DIA were analysed by gas chromatography (Formica, G and Giannone, C., 1986). Results are shown in Table 6.

Table 6: DIA Residues in selected pig tissues from the 10-day withholding time

	DIA Residue (mg/kg equivalents avilamycin)			
Animal No.	Muscle	Liver	Kidney	Fat
130	--	0.10	< 0.1	NDR
135	--	0.12	< 0.1	NDR
137	--	0.17	< 0.1	NDR
Mean	--	0.13	< 0.1	NDR

Approximately 50% of TRR in liver was due to DIA-related residues. DIA-related residues were detected in kidney, but below the limit of quantification (LOQ < 0.1 mg/kg). No DIA residues were observed in fat (< 0.1 mg/kg). Liver results are presented in Table 7.

Table 7: Pig liver extraction results

Dosing Interval	Mean (n=3) Percent of Radioactivity	
Days	Acetone	Unextracted
4	79.5	20.5
7	82.2	17.8
10	73.1	26.9

About 18 - 27% of radioactivity was not extractable into acetone for the 4, 7 and 10 day liver samples. Statistical analysis of the extraction data indicated no significant difference between un-extracted radioactivity through 10 days of treatment.

A steady-state, tissue residue study using uniformly labelled ^{14}C avilamycin was conducted by Magnussen, et al., 1987. Six crossbred pigs, four barrows and two gilts, weighing approximately 44 kg each were fed at 12-hour intervals for either ten or fourteen days with a ration containing a nominal concentration of 60 mg of ^{14}C-avilamycin/kg of feed (equivalent to 60 mg activity/kg and to 3.6-4.8 mg/kg bw/day). Each day, animals received an amount of ration equal to 4% of their body

weights. Groups of three animals were killed after ten days and fourteen days on treatment. Muscle, liver, kidney, and fat were collected for radiochemical analysis. Liver from each animal was extracted to determine the concentration of non-extractable radioactivity and to characterize the extractable radioactivity. Radioactivity in fat was also characterized. Total radioactivity residues in tissues are presented in Table 8.

Table 8: Total radiolabel residues (TRR) in pig tissues

Dosing Interval	TTR (mg/kg equivalents avilamycin)			
Days	Muscle	Liver	Kidney	Fat
10	0.09	0.55	0.32	0.26
14	0.14	0.66	0.34	0.55

Radioactive tissue residues are higher in this study than the other two ^{14}C studies because the avilamycin molecule was more uniformly labelled over all rings with ^{14}C for this study. The ^{14}C label in the avilamycin for the other two studies was primarily (85%) in the DIA ring. One-way analysis of variance (ANOVA) indicated no difference between 10 or 14 days for muscle, liver or kidney total radioactive residues. Only the fat radioactive residues were significantly different at 10 and 14 days ($P < 0.05$). Non-extractable liver residues were 33 - 37% of total liver residues and were not different in the 10- and 14-day treatment groups as are shown in Table 9.

Table 9: Percent extraction of radioactivity from pig livers

	10-day Group			14-day Group		
Animal No.	961	960	957	954	955	959
Acetone Extract	34	32	34	32	33	29
Methanol Extract	25	24	26	25	26	28
Acetone/water	7	7	6	8	6	6
Pellet	34	37	33	34	35	37

The GC analysis shown that extractable liver radioactivity consisted of several minor metabolites (<0.1 mg/kg). Flambic acid was present at concentrations up to 0.04 mg/kg. Parent ^{14}C-avilamycin concentrations were less than 0.01-0.02 mg/kg.

Chickens

In a GLP-compliant conducted study (Dalidowicz, 1986), twelve seven-week-old broiler-type chickens, six male and six female, were fed a standard broiler finishing ration containing 14.16 mg of [DIA^{14}C] avilamycin per kilogram of feed (equivalent to 15 mg of activity /kg and equal to 3 mg/kg bw/day) for either four, seven, or ten days. Medicated ration and water were provided *ad libitum* throughout the dosing phase. At the end of each designated dosing period, two birds of each sex were deprived of food and water for six hours and then killed. Samples of muscle, liver, abdominal fat, kidney and skin with subcutaneous fat were collected for radiochemical analysis. Results are shown in Table 10.

Table 10: Total radiolabel residues (TTR) in chicken tissues

Tissue	TTR (mg/kg equivalents of Avilamycin)			
	Method LOD	4 day	7 day	10 day
Muscle	0.01	< 0.01[1]	NDR[2]	NDR
Liver	0.01	0.03	0.04	0.02
Skin	0.01	0.02[3]	0.01[3]	0.02[3]
Fat	0.01	0.01[4]	0.03	0.03[3]
Kidney	0.02	NDR	NDR	NDR

[1:] Three of four individuals below LOD; LOD value substituted for NDR of individuals
[2:] NDR: no detectable residue (less than LOD)
[3:] One of four individuals below LOD; LOD value substituted for NDR of individuals
[4:] Study director excluded one of four samples as a statistical outlier.

Reliable detection and quantitation were demonstrated only for 0.025 mg/kg. After ten days dosing, the mean total radiolabel residues in skin, liver, and fat expressed as avilamycin equivalents, were 0.02, 0.02, and 0.03 mg/kg, respectively. Muscle and kidney samples contained no detectable radiolabel residues. Steady-state concentrations of radioactivity were attained in all tissues within four to seven days after the initiation of dosing.

In another GLP-compliant study, twenty-four Highline W-36 laying hens were fed rations containing 30 mg/kg [U^{14}C] avilamycin for fourteen days (Sweeney, et al., 1997). Eggs were collected daily throughout the study. At slaughter, liver, kidney, muscle, fat, skin/fat, and bile were collected. The tissues were assayed for total radioactivity by solubilization and liquid scintillation counting. Results are summarized in the Table 11.

Table 11: Total radiolabel residues in chicken tissues

	TTR (mg/kg equivalents of Avilamycin)					
	Liver	Kidney	Muscle	Skin/fat	Fat	Bile
Mean(n=7)	0.08	0.07	NDR	NDR	0.03	3.5

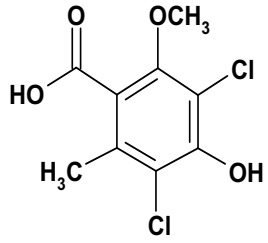
NDR: No Detectable Residues

Eggs from study day five, ten, twelve, and fourteen were separated into yolk and albumin and analysed for radioactive residues. Residues in albumin were not detectable (<0.07 mg/kg), while the residues in yolk were on the average 0.2 mg/kg at 10 days, 0.21 mg/kg at 12 days and 0.22 mg/kg at fourteen days. One hen had significantly higher liver, kidney, and yolk residues than the other six treated hens. The higher residue values in this hen were attributed to animal-to-animal variation.

Turkeys and Rabbits

No radiolabelled residue depletion studies data are available.

Avilamycin is not a suitable marker residue because it is not detected in tissues of pigs and chickens. Flambic acid, the major metabolite, is not a suitable marker residue because it does not have a reference standard available. Dichloroisoeveninic acid (DIA) is a moiety present in avilamycin, along with flambic acid and other possible metabolites. Measurement of DIA following extraction and hydrolysis of DIA-containing fractions or metabolites provides a satisfactory method for measuring residues of avilamycin, as studies have demonstrated measurable amounts of DIA in liver and kidney. DIA is a useful marker residue because it is not a common chemical structure and where it can be found in related substances, none of them are veterinary drugs. The DIA concentration may be reported as avilamycin equivalents by multiplying the DIA concentration by the molar ratio of avilamycin/DIA (5.6:1).



Dichloroisoeverninic acid

Residue Depletion Studies with Unlabelled Drug

Residues in Tissues

Pigs

A GLP-compliant residue study in young pigs was submitted (Eichmeier, et al, 2006a). Twelve crossbred commercial pigs (plus 1 male and 1 female as controls) weighing about 9 to 15 kg were fed *ad libitum* a commercial diet containing avilamycin at a nominal concentration of 150 mg/kg feed for 21 consecutive days (equal to 9-12 mg/kg bw/day). At the end of the 21-day exposure period, animals were euthanized at withdrawal intervals of 0, 6, and 24-hours (n=4 per group, 2 males and 2 females). Samples of liver, kidneys, muscle and skin with fat were collected. Avilamycin residues were analyzed as DIA by a LC-MS-MS validated method (Eichmeier, 2006a) and also by a microbiological assay (Eichmeier, et al, 2006, Appendix G). The LC-MS-MS method requires a tissue hydrolysis step to yield dichloroisoeverninic acid. Results were reported as avilamycin equivalents by multiplying the determined DIA concentration by the molar ratio (5.6:1). The mean residue data are summarized in the Table 12. Residues were only detected in liver tissue.

Table 12: Equivalent avilamycin residues in pig tissues

	Mean Equivalent Avilamycin Residues (µg/kg) ± SD[1]			
	Liver	Kidney	Muscle	Fat/Skin
Control	< LOD[2]	<LOD	<LOD	<LOD
0 hr	103 ± 26	< 28 [3]	<LOD	<LOD
6 hr	42 ± 9	< 28 [3]	<LOD	<LOD
24 hr	< 32[4]	ND	<LOD	<LOD

[1:] Mean ± standard deviation
[2:] Limit of Detection in avilamycin equivalents/kg tissue: Liver (9.0 µg/kg); Fat/Skin (5.5 µg/kg); Muscle (4.2 µg/kg); Kidney (3.3 µg /kg)
[3:] 28 µg/kg = 5.0 µg/kg DIA
[4:] n = 1, other 3 samples < 28 µg/kg

For the microbiological assay samples were extracted with acetone, purified and the organic phase analyzed by thin layer-chromatography (TLC) on silica gel plates. Antibiotic activity was assayed using *Micrococcus luteus* ATCC No. 10240 as the assay organism. The pig muscle, liver, and skin/fat tissues for zero hour and 6 hr withdrawal times showed no response on the assay plates at a limit of detection of 5 µg/kg tissue. The 24-hr samples were not assayed for antimicrobial activity.

The results of this study showed that DIA was quantifiable in pig liver at zero and 6 hr withdrawal time and declined by more than half in 6 hours. After 24 hours, the residues were below or near 28 µg avilamycin/kg tissue. DIA residues were detected, but not quantifiable, in kidney at 0 and 6 hr withdrawal, and were not detected after 24 hours. No residues were detected in muscle or fat/skin samples at any time. No antimicrobial activity was detected in any tissue (LOD = 5 mg/kg) indicating that DIA detected in liver and kidney was due to inactive metabolites of the drug.

A non GLP-compliant study to determine microbiological activity of avilamycin residues was submitted (Asanuma, et al., 1987a). A preparation of 10% avilamycin/mg (EL-750) was administered to castrated male pigs from 28 to 84 days orally by medicated feed at concentrations of 40 or 400 mg/kg feed (two groups of 8 pigs, one control). Non-medicated feed was provided during withdrawal. The pigs were allowed free access to the feed. Liver, kidney, muscle, fat tissues and small intestine were collected at 42 days (two animals per group) and after 84 days (two animals at each time, 0.1 and 3 days withdrawal). The avilamycin residues were analysed by the microbiological assay method with *Micrococcus flavus* (LOD = 25 µg/kg). Avilamycin was not detected in major organs or tissue for all sampling points including during medication.

In a previously reported non GLP-compliant study, the microbiological activity was studied by Morimoto et al., 1986a. Breeding piglets (22 boars and 22 sows) at approximately 30 days old were fed with medicated feed containing avilamycin (40, 200 or 400 mg/kg) for 12 weeks followed by a 7-day withdrawal. The avilamycin preparation was the same used in the previous mentioned study (EL-750. 10% avilamycin/mg). Plasma, liver, kidney, muscle, fat, and small intestine was collected from groups of 1 male and 1 female at 6 weeks and 12 weeks at 0-hour withdrawal and at 1, 3, 5 and 7days withdrawal time. The residues were analyzed using the TLC/microbiological assay based on inhibition of *Micrococcus flavus* (LOD = 25 µg/kg). No microbiologically active residues were observed in any samples of plasma, muscle, liver, kidney, or fat (25 µg/kg was detected in two small intestine samples from the 200 mg/kg treatment group, one at 6 weeks and one at 12 weeks; 27 µg/kg was detected in a small intestine sample of the 400 mg/kg group at 6 weeks and 30 µg/kg at 12 weeks). No other residues were detected. The authors concluded that EL-750 is not readily absorbed and only very small amounts of avilamycin are found in tissues, even at a dose of 400 mg/kg in the feed for 12 weeks.

Two non GLP-compliant studies simulating the commercial pig industry were reported.

In the first study (West and Wellenreiter, 1983), grower-finisher pigs (2 male, 4 female) were fed standard rations containing 0 or 40 mg/kg of avilamycin for a period of 99 days. The pigs were sacrificed after a zero-day (six-hour) or a one-day (30-hour) withdrawal period. Using the bio-autographic technique with *Micrococcus flavus* as the indicator organism, no microbiologically active residues were detected in the muscle, liver, kidney or fat tissues from any of the six treated or three control pigs analysed at a detection limit of 0.05 mg/kg.

In the second study (West, et al, 1983), starter pigs (3 male, 3 female) were fed a standard ration containing 0 or 200 mg/kg of avilamycin for a period of 56 days. The pigs were sacrificed after a zero-day (six-hour) withdrawal time. Using the microbiological assay, no microbiologically active residues were detected in the muscle, liver, kidney or fat tissues from any of the pigs analysed at a detection limit of 0.05 mg/kg.

Chickens

In a GLP-compliant residue study, a commercial breed of broiler chickens (15 males and 15 females plus 10 males and 10 females as controls) approximately two weeks old weighing from 339-541 g were fed *ad libitum* a commercial diet containing avilamycin at a nominal concentration of 150 mg/kg feed for 21 consecutive days, equivalent to 30 mg/kg bw/day (Eichmeier, 2006b). At the end of the 21-day exposure period, animals were euthanized at withdrawal intervals of 0, 6, and 24-hours (n=6 per group, 3 males and 3 females). Samples of liver, kidneys, muscle and skin/fat (subcutaneous) were collected. Avilamycin residues were analyzed as DIA by the LC/MS/MS method (Eichmeier, 2006b) and also by the microbiological assay (Eichmeier, et al., 2006, Appendix G) as previously described for the study with pigs (Eichmeier, et al., 2006a).

The DIA moiety of avilamycin was quantifiable in chicken liver at zero time withdrawal and declined to below or near 28 µg avilamycin/kg tissue within 6 hours. After 24 hours, the liver residues were

below 28 µg avilamycin/kg. DIA residues were detected, but not quantifiable, in kidney and skin/fat at 0 and 6 hours withdrawal, and were not detected after 24 hours. Half of the skin/fat and kidney samples had no detectable residues after 6 hours withdrawal. No DIA residues were detected in muscle samples at any time. No antimicrobial activity was detected in any tissue from the 0-hr and 6-hr treated groups (LOD = 5µg/kg), with the exception of two skin/fat samples from the 6-hr treated group that were attributed to laboratory contamination. The 24-hr samples were not assayed for antimicrobial activity. No antimicrobial activity was detected in any other tissue (LOD = 5 µg/kg). Therefore, DIA detected in liver and kidney was due to inactive metabolites of the drug. Results for the LC-MS-MS analysis are shown in Table 13.

Table 13: Avilamycin equivalent residues in chicken tissues

	Mean Equivalent Avilamycin Residues (µg/kg) ±SD[1]			
	Liver	Kidney	Muscle	Fat/Skin
Control	<LOD[2]	<LOD	<LOD	<LOD
0 hr	67 ±32	< 28[3]	<LOD	< 28
6 hr	< 30[4]	< 28[5]	<LOD	< 28[2]
24 hr	< 28[2]	<LOD	<LOD	<LOD

1: Mean ± Standard Deviation
2: Limit of Detection (in avilamycin equivalents/kg tissue): Liver (3.0 µg/kg); Fat/Skin (5.0 µg/kg); Muscle (4.4 µg/kg); Kidney (4.9 µg/kg)
3: 5.0 µg/kg DIA =28 µg avilamycin/kg tissue
4 n = 1, other 5 samples < 28µg/kg
5: n=3 at <28µg/kg , n = 3 < LOD

A non GLP-compliant study to determine microbiological activity of avilamycin residues was submitted (Asanuma, et al., 1987b). A preparation of 10 % avilamycin/mg (EL-750) was administered to chickens (40-47 weeks old, 920-1090 g) for 56 days using medicated feed at concentrations of 10 or 200 mg/kg (60 chickens per group). The birds were allowed free access to the feed. The residues of avilamycin were analysed by the microbiological assay method with *Micrococcus flavus*. Avilamycin was not detected in major organs or tissue for all sampling points including the medication period, thus, avilamycin is not readily absorbed.

In an earlier non GLP-compliant study, the microbiological activity was studied by Morimoto, et al., 1986b. A preparation of 10 % avilamycin/mg (EL-750) was administered to broiler chickens from day 1 to 84 weeks orally by medicated feed at concentrations of 20, 100 or 200 mg avilamycin/kg feed (3 groups of 6 males and 6 females, one control group). Non medicated feed was used during the 7 day withdrawal time. Plasma, liver, kidney, muscle, fat, and small intestine were collected at 28 days, 56 days (0-hour withdrawal), and at 1, 3, 5 and 7 days withdrawal. The residues were analyzed using the TLC/microbiological assay based on *Micrococcus flavus* as the indicator organism. No microbiologically active residues were observed in any samples of plasma, muscle, liver, kidney, or fat. (LOD= 0.05 µg/kg).

In a third non GLP-compliant study, broiler chickens (six treated plus two control birds) were fed standard rations containing 0 or 20 mg of avilamycin/kg of feed for a period of 56 days (West, et al., 1983). The chickens were sacrificed after a zero-day (six-hour) withdrawal. Using the bio-autographic technique with *Micrococcus flavus* as the indicator organism, no microbiologically active residues were detected in the muscle, liver, kidney, or skin with adhering fat tissues from any of the chicken.

Turkeys

A GLP-compliant residue study in turkeys was submitted (Eichmeier, et al., 2006c) to demonstrate the applicability of the routine analytical residue method for the determination of avilamycin and dichloroisoeverninic acid-containing metabolites in turkey tissues. Domesticated turkeys (*Melleagris*

gallopago) (3 males and 2 females plus 1 male and 1 female as controls) approximately 8 weeks old weighing 2.9 to 5.2 kg were fed *ad libitum* a commercial diet containing avilamycin at a nominal concentration of 150 mg/kg feed for 7 consecutive days (equivalent to 30 mg/kg bw/day). At the end of the 7-day exposure period, birds were euthanized at 0 withdrawal time. Samples of liver, kidneys, muscle and skin with fat were collected. Avilamycin residues were analyzed as DIA by the LC/MS/MS method validated for chickens with applicability demonstrated for turkey tissues (Eichmeier, et al., 2006c, Appendix F) and also by a microbiological assay (Eichmeier, et al., 2006, Appendix G). Results for the LC/MS/MS analysis are shown in Table 14.

Table 14: Avilamycin equivalent residues in turkey tissues

	Mean Equivalent Avilamycin Residues (µg/kg) ±SD[1]			
	Liver	Kidney	Muscle	Fat/Skin
Control	< LOD[2]	<LOD	<LOD	<LOD
0 hr	117±47	< 28 [3]	<LOD	61±30

[1:] Mean ± standard deviation
[2:] Limit of Detection in avilamycin equivalents/kg tissue: Liver (3.0 µg/kg); Fat/Skin (5.0 µg/kg); Muscle (4.4 µg/kg); Kidney (4.9 µg/kg)
LOD values determined for chicken blank tissues were used.
[3:]5.0 µg/kg DIA = 28 µg avilamycin/kg tissue

Avilamycin residues were below the LOD in muscle and below 28 µg avilamycin/kg in kidney. In liver samples, quantifiable DIA levels were found in all five treated turkeys, with values of 67.6 to 195 µg/kg avilamycin equivalents at zero hour withdrawal. In fat/skin samples, quantifiable DIA levels were found in four of the five treated turkeys, at 37.3-105 µg/kg avilamycin equivalents. No microbiological activity was detected in kidney, muscle, and liver tissues (limit of detection is 5 µg/kg). The skin/fat tissues for four animals contained detectable residues at or below 25 µg/kg. The residues at 0 hour withdrawal time were very low in liver and fat/skin and below the LOD of 28 µg/kg in muscle and kidney. Antimicrobial activity was not found in liver, kidney and muscle samples. Skin/fat samples were positive for antimicrobial activity which may have been the result of contamination during the in-life phase. The LC-MS/MS method for DIA is capable of detecting and quantifying incurred residues in tissues from turkeys treated with avilamycin in the feed.

Rabbits

A GLP-compliant residue study in rabbits was submitted (Eichmeier, et al., 2006d) to demonstrate the applicability of the routine analytical LC-MS-MS for DIA residues in rabbit tissues. Rabbits (*Orytolagus cuniculus*) (3 males and 2 females plus 1 male and 1 female as controls), approximately 7 weeks old and weighing about 1.1 to 1.5 kg, were fed *ad libitum* a commercial diet containing avilamycin at a nominal concentration of 125 mg/kg feed for 7 consecutive days (equivalent to 7.7 mg/kg bw/day). Animals were euthanized at zero withdrawal time. Avilamycin residues were analyzed as DIA by the LC/MS/MS method validated for pigs (Eichmeier, 2006c, Appendix F) and also by a microbiological assay (Eichmeier, et al., 2006, Appendix G). Results are shown in Table 15.

Table 15: Avilamycin equivalent residues in rabbit tissues

	Mean Equivalent Avilamycin Residues (µg/kg) ±SD[1]			
	Liver	Kidney	Muscle	Fat
Control	< LOD[2]	<LOD	<LOD	<LOD
0 hr	124 ±22	284 ±52	< 28 [3]	< 28 [3]

[1] Mean ± standard deviation
[2] Limit of Detection in avilamycin equivalents/kg tissue: Liver (9.0 µg/kg); Fat/Skin (5.5 µg/kg); Muscle (4.2 µg/kg); Kidney (3.3 µg/kg)
LOD values determined for pig blank tissues were used.
[3] 5.0 µg/kg DIA = 28 µg avilamycin/kg tissue

In all five treated rabbits, the equivalent avilamycin levels were below 28 µg/kg in muscle and fat at zero hour withdrawal time. In liver and kidney samples, quantifiable DIA levels were found in all five treated rabbits at 93 – 145 µg/kg and 228 – 352 µg/kg avilamycin equivalents, respectively. No microbiological response was detected in rabbit kidney, muscle, fat and liver tissues on the assay plates at a limit of detection of 5 µg/kg tissue. The residues at zero withdrawal time were very low in liver and kidney and below the 28 µg/kg in muscle and fat. The routine analytical method (LC-MS/MS for DIA) was capable of detecting and quantifying incurred residues in tissues from rabbits treated with avilamycin in the feed. Antimicrobial activity was not found in liver, fat, kidney and muscle samples.

METHODS OF ANALYSIS

Pigs

An analytical method validated for the determination of avilamycin in pig tissues (muscle, fat, liver and kidney) was submitted (Eichmeier, 2006b). Avilamycin and/or its metabolites containing dichloroisoeverninic (DIA) were extracted from homogenized tissues using acetone. Following centrifugation and evaporation of the solvent, the extracted residues were hydrolyzed at about 70°C for 2 hr in 1 N NaOH. The hydrolysate was acidified to pH 1 with phosphoric acid and partitioned with ethyl acetate. An external standard was added (Dicamba) to appropriate samples and the hydrolysate was purified by alumina solid phase extraction (SPE). DIA was eluted from a SPE cartridge using 5% formic acid in acetonitrile. The eluate was evaporated to dryness and reconstituted in methanol for LC-MS-MS analysis. The DIA concentration was measured by gradient HPLC with mass spectrometric detection using negative-ion electrospray ionization mass spectrometry with selected-ion monitoring of the molecular ions of DIA and the external standard ($M+H^+$). HPLC was performed using a Synergi Polar-RP 80A column (75 x 4.6 mm, 4µm, injection volume 10-25 µl, flow rate 0.6 ml/min) with a solvent gradient (0.1% formic acid in water-methanol, run time 8 minutes). A Phenomenex Security Guard C18 (4.0 x 3.0 mm) was employed. For the MS detection the instrument acquisitions were: Detector PE Sciex API 3000, APCI, negative mode, Ion Spray Voltage -4200, Scan Dwell time 250ms for both DIA and Dicamba, Turbo gas temperature was 550 ºC. A MRM procedure was applied and the following transitions were monitored: m/z 249.0 →190.0 and 219.2→ 175.0 for DIA and the external standard respectively.

Calibration curves are constructed by weighted linear regression using the DIA peak area or the ratio of the peak area of the DIA to that of the added external standard, if used. The DIA concentration was converted to avilamycin equivalents by multiplying the DIA concentration by the molar ratio of avilamycin/DIA (5.6:1).

Linearity: The linear range is from 28 to 3000 ng/ml of avilamycin equivalents (DIA nominal linear range is from 5 to 550 ng/ml). Duplicate calibration curves were use to generate the linear regression curve for each tissue with nominal calibration standards at 5, 10, 25, 50, 100, 150, 200, 350 and 550 ng/ml. The correlation coefficient (r) values were > 0.990 in all cases.

Accuracy/Recovery/Precision (repeatability): Within laboratory data at three concentrations: (300 µg DIA/kg for liver, 200 µg DIA/kg for kidney, 100µg DIA/kg for skin/fat and 50µg DIA/kg for muscle) were used to determine the accuracy and precision of the measured concentration of avilamycin. Replicates (n=3) of test portions of tissue at each avilamycin concentration were analyzed each day for three days. Repeatability (n=6) was used as one of the within laboratory day runs. Within run repeatability precision was from 9.8 to 14.6%. Within laboratory precision was between 8.4 and 14.6%. Accuracy, determined as percent recovery from tissues fortified with avilamycin was between 79 and 105%.

Limit of Detection: Blank matrix samples were assayed as part of the within-laboratory analytical batches and additional blank matrix samples (>10) were assayed for each tissue type from a minimum of 2 different animals. The response from 20 or more blank control test portions for each tissue plus

three times the standard deviation determined the limit of detection for each tissue. The claimed avilamycin limits of detection were 5.5µg/kg DIA for fat/skin, 4.2µg/kg for muscle, 3.3µg/kg for kidney and 9.0µg/kg for liver.

The Committee reconsidered the data provided and calculated the LOQs considering the representative chromatograms of typical LC-MS-MS spectra of the extracted fortified samples for pigs using the criteria of signal to noise ratio equal to 10.

Limit of Quantification: The sponsor adopted the LOQs as the minimum concentration in fortified samples that were shown to satisfy the criteria for recovery and precision, – i.e., the lowest concentration on the calibration curve and rounding these values (150µg DIA/kg for liver, 100µg DIA/kg for kidney, 50µg DIA/kg for skin/fat and 25µg DIA/kg for muscle). Repeatability measurements at concentrations noted below using signal to noise ratios were used to estimate LOD and LOQ at avilamycin equivalents of 100µg/kg in muscle; 750µg/kg in liver; 500µg/kg in kidney; and 250µg/kg in skin/fat. Results are tabulated in table 16.

Table16: LOD and LOQ determinations for pig tissues

Tissue	Avilamycin (µg/kg)	LOD (µg/kg)	LOQ (µg/kg)
Muscle	100	7.2	24
Liver	750	3.0	10
Kidney	500	1.0	3.3
Skin/Fat	250	6.7	22.4

Selectivity, Specificity and Carry-Over: These parameters were evaluated by extracting and analyzing individual blank pig liver, kidney, muscle and fat/skin samples. No significant response at the retention times of DIA or the external standard (Dicamba), were noted in the tissue blanks. Specificity was also examined by separately analyzing medicated feed additives of monensin, tylosin, tilmicosin, nicarbazin, narasin, salinomycin and clopidol. These reference compounds were processed by the avilamycin method procedure through to analysis by HPLC-MS/MS. None of the medicated feed additives showed a response at the retention time of DIA or Dicamba. Carry-over was evaluated by placing vials of solvent blank (methanol) at several locations in the analysis set after a high calibration standard sample. No carry over was observed in the solvent blank samples.

Robustness: Examination of different lots of HPLC columns and the effect of variation of pH (0.2 pH units) after the method hydrolysis step in DIA extraction were used to determine the robustness using liver extracts. The mean accuracy values of the tested extracts were within 20% of each other.

Stability: DIA solutions in reconstituted solvent for HPLC-MS/MS analysis for tissues are stable at 4 to 8 ºC for at least 7 days. Avilamycin fortified tissue samples are stable for at least 9 months at -70ºC. Liver, kidney and fat/skin extracts are stable for at least 7 days at room temperature and muscle extracts for at least 19 days. DIA and Dicamba in methanol stored at 4-8ºC are stable for at least 8 and 9 months respectively.

Chickens

The analytical validated method for the determination of avilamycin in pig tissues (muscle, fat, liver and kidney) for use with chicken tissues was submitted (Eichmeier, 2006a). Identical clean-up steps were used, HPLC/MS/MS conditions and construction of calibration curves described for pig samples were applied.

Linearity: Results are described in the pig tissue method.

Accuracy/Recovery/Precision (repeatability): Within laboratory data at 0.5 MRL, MRL and 2xMRL of the sponsor proposed MRLs (300 µg/kg for liver, 200 µg/kg for kidney, 100 µg/kg for skin/fat and 50 µg/kg for muscle) were used to determine the accuracy and precision of the measured concentration of avilamycin. Replicates (n=3) of test portions of tissue at each avilamycin concentration were analyzed each day for three days. Repeatability (n=6) was used as one of the within laboratory day runs. Within run repeatability precision ranged from 7.9 - 13.3%. Within laboratory precision ranged between 7.5 to 20.6%. Accuracy, determined as percent recovery from tissues fortified with avilamycin ranged between 82 - 105%.

Limit of Detection: Blank matrix samples were assayed as part of the within-laboratory analytical batches and additional blank matrix samples (11) were assayed for each tissue type from a minimum of 2 different animals. The response from 20 or more blank control test portions for each tissue plus three times the standard deviation determined the limit of detection for each tissue. The claimed avilamycin limits of detection were 5.0 µg/kg for fat/skin, 4.4 µg/kg for muscle, 4.9 µg/kg for kidney and 9.0 µg/kg for liver.

The Committee reconsidered the data provided and calculated the LOQs considering the representative chromatograms of typical LC-MS-MS spectra of the extracted fortified samples for chickens using the criteria of signal to noise ratio equal to 10.

Limit of Quantification: The sponsor adopted the LOQs as the minimum concentration in fortified samples that were shown to satisfy the criteria for recovery and precision, – i.e., the lowest concentration on the calibration curve and rounding these values (150 µg DIA/kg for liver, 100 µg DIA/kg for kidney, 50 µg DIA/kg for skin/fat and 25 µg DIA/kg for muscle). It was deemed that a more appropriate measure of limit of quantification was the comparison of signal to noise ratios of typical LC-MS-MS spectra. Repeatability measurements at concentrations noted below were used to estimate LOD and LOQ as avilamycin equivalents of 100µg/kg in muscle; 750 µg/kg in liver; 500 µg/kg in kidney; and 250 µg/kg in skin/fat. Results are tabulated in table 17.

Table17: LOD and LOQ determinations for chicken tissues

Tissue	Avilamycin (µg/kg)	LOD (µg/kg)	LOQ (µg/kg)
Muscle	100	5.7	18.8
Liver	750	9.1	30.4
Kidney	500	6.7	22.4
Skin/Fat	250	5.6	18.7

Selectivity, Specificity, Carry-Over and Robustness: These parameters were evaluated with identical procedures as those employed in the pig tissue method with identical results.

Stability: DIA tissue extracts are stable at 4 to 8 ºC for at least 7 days in muscle, liver and fat/skin and for at least 6 days in kidney. Avilamycin - fortified tissue samples are stable at -70 ºC for at least 7.5 months in liver, muscle and skin/fat and for at least 9 months in kidney tissue. Liver and fat/skin extracts are stable for at least 7 days and kidney and muscle for at least 6 days at room temperature.

Turkeys

The same analytical method for the determination of avilamycin in chicken tissues was employed and its applicability in turkey tissues was demonstrated (Eichmeier, et al., 2006c, Appendix F). The method was capable of detecting and quantifying incurred residues in tissues from turkeys treated with avilamycin in feed.

Linearity of the DIA calibration curves was acceptable with correlation coefficient (r) values for this study ranging from 0.9959 to 0.9989. Recovery samples were analyzed at tissue fortification levels of 50 µg/kg for each tissue; acceptable recoveries were from 72 to 103%. LOD and LOQ were nearly equivalent to those adopted for chicken tissues.

Rabbit

The same analytical method for the determination of avilamycin in pig and chicken tissues was demonstrated (Eichmeier et al., 2006d, Appendix F). The method was capable of detecting and quantifying residues incurred in tissues from rabbits treated with avilamycin in the feed. Linearity of the DIA calibration curves was acceptable and correlation coefficient (r) values for this study were 0.9957 to 0.9985. Recovery samples were analyzed at tissue fortification levels of 50 µg/kg for each tissue; acceptable recoveries ranged from 81 to 110%. The LOD and LOQ were nearly equivalent to that determined for chicken tissues

APPRAISAL

Avilamycin has not been previously evaluated by the Committee. Avilamycin is an orthosomycin antibiotic complex primarily active against Gram-positive bacteria. The major fermentation product consists of avilamycin A and avilamycin B while 15 minor factors have been identified. Avilamycin is intended for use only as a veterinary medicine in chickens, turkeys, pigs and rabbits to control bacterial enteric infections at a dose of 100 mg/kg feed for 21 days. In rabbits it is administered orally at a dose of 80 mg/kg feed for 28 days. No classical pharmacokinetic studies were conducted in any species with avilamycin because avilamycin is not detectable in plasma following oral administration of avilamycin in feed. Metabolism and residue studies in pigs, poultry and the rat were conducted using radiolabelled material. Where avilamycin residues were measured in blood, serum or plasma following oral doses, they were below the limits of detection.

In rats, less than one percent of the oral dose was eliminated in the urine while 80 - 104% was recovered in the faeces. Similar results were observed in food animal species. For example, when avilamycin is administered orally to pigs, 92 - 93% of the residues are recovered in the faeces and 5-8% in the urine. Similar results were found for chickens. Pharmacokinetic data in turkeys and rabbits are not available. However, pharmacokinetic data in rats, pigs and chickens are highly consistent. Owing to the similarity of species, pharmacokinetic data in chickens may be applied to turkeys.

The metabolite pattern in urine and faeces of treated pigs was essentially the same as the pattern for rats. Parent avilamycin constituted less than 10% of the faecal radioactivity in pigs. Similarly, the metabolite profiles in livers of treated rats and pigs were essentially the same. Parent avilamycin concentrations in rat and pig livers were less than 0.05 mg/kg. The most abundant metabolite was flambic acid. The pattern of minor metabolites was similar, but none of the minor metabolites were sufficiently abundant for identification. Characterization of residues in fat samples from pigs demonstrated that almost all radioactivity in fat was due to its incorporation into the endogenous fatty acids. No metabolism data are available on turkeys or rabbit.

Dichloroisoeverninic acid (DIA) is a moiety present in avilamycin, flambic acid and other possible metabolites that can be released by hydrolysis of avilamycin residues. DIA is proposed as the marker residue. The DIA concentration may be reported as avilamycin equivalents by multiplying the determined DIA concentration by the molar ratio of avilamycin/DIA of 5.6:1. As noted below, the only tissue with measurable residues at six hour withdrawal times is liver, and is the only possible target tissue.

Three GLP-compliant radiolabelled residue studies in pigs were submitted. Two of them used avilamycin labelled in the DIA moiety [DIA-^{14}C], and the third used uniformly labelled [U-^{14}C] avilamycin. One GLP -compliant [DIA-^{14}C] radiolabelled study in chickens was submitted. In all

studies, animals were slaughtered at a practical zero-time withdrawal of 6 h after the final feeding of medicated ration.

In the first study, pigs fed a ration containing [^{14}C-DIA]avilamycin in feed at 12 hour intervals for 7 days, the concentrations of avilamycin-related radioactivity in liver and muscle declined to non-detectable levels within 3 days after the termination of dosing, whereas concentrations in kidney declined to near non-detectable levels within 5 days after the termination of dosing (LOD = 0.025 mg/kg). Radioactivity in fat showed a much slower rate of decline due to [^{14}C]avilamycin being incorporated into the fatty acid fraction.

In the second study in pigs using the same radiolabelled compound fed at 12 hour intervals for 4, 7 or 10 days, total radioactive residues in liver, muscle, fat and kidney, expressed as avilamycin equivalents, were 0.22, 0.02, 0.12 and 0.10 mg/kg, respectively. Steady-state concentrations of radioactivity were attained in muscle, liver and kidney within 4 days after the initiation of dosing. A steady-state concentration was not attained in fat; residues were 0.12 mg/kg at 10 days. No residues of parent avilamycin were detected in pig kidney or fat analysed by thin-layer chromatography bio-autography after 10 days of treatment, and only traces were detected in liver. Muscle was not assayed because of very low amounts of radioactivity (LOQ <0.05 mg/kg). Approximately 50% of total radiolabelled residues in liver were DIA-related residues. DIA-related residues were detected in kidney, but were less than the LOQ (<0.1 mg/kg). No DIA residues were observed in fat (<0.1 mg/kg).

In the third study, pigs were dosed with [U-^{14}C]avilamycin at 12-h intervals for either 10 or 14 days. After 10 days of treatment, total radioactive residues expressed as avilamycin equivalents in liver, fat, muscle and kidney were 0.55, 0.26, 0.09 and 0.32 mg/kg, respectively. There was no statistical difference in total radioactive residues in muscle, liver or kidney at 10 or 14-day dosing times. Only the radioactive residues in fat were significantly different between 10 and 14 days ($P < 0.05$). The gas chromatographic analysis showed that extractable liver radioactivity consisted of several minor metabolites (<0.1 mg/kg). Flambic acid was present at concentrations up to 0.04 mg/kg. Parent [^{14}C]avilamycin concentrations were less than 0.01-0.02 mg/kg.

Total residues in broiler chickens fed a standard broiler finishing ration containing [DIA-^{14}C] avilamycin in feed for up to 10 days. Total residues at ten days, expressed as avilamycin equivalents, in skin, liver and fat were 0.02, 0.022 and 0.03 mg/kg, respectively. Muscle and kidney samples contained no detectable radiolabel residues. Steady-state concentrations of radioactivity were attained in all tissues within 4-7 days after the initiation of dosing.

No radiolabelled depletion studies on turkeys or rabbits are available.

One GLP-compliant non-radiolabelled residue depletion study was provided for pigs. Pigs fed a commercial diet containing avilamycin *ad libitum* for 21 consecutive days. Using a LC/MS/MS validated method and also by a microbiological assay, the DIA moiety of avilamycin was quantifiable in pig liver at 0 and 6 hours withdrawal. After 24 hours, the residues were below 28 µg avilamycin equivalents/kg tissue. DIA residues were detected, but not quantifiable, in kidney at 0 and 6 hour withdrawal and were not detected after 24 hours. No residues were detected in muscle or fat/skin samples at any time. No antimicrobial activity was detected in any tissue by an inhibition assay using *Micrococcus luteus* as the indicator organism. Thus, DIA residues detected in liver and kidney are due to microbiologically inactive metabolites of the drug.

In a non-radiolabelled broiler chickens study, birds were fed a commercial diet containing avilamycin *ad libitum* for 21 consecutive days (equal to 30 mg/kg bw/day). After a 21-day exposure period, DIA was quantifiable in chicken liver at 0 time withdrawal and declined to 28 µg avilamycin equivalents/kg tissue or less within 6 hours. DIA residues were detected, but not quantifiable, in kidney and skin/fat at 0 and 6 hour withdrawal and were not detected after 24 hours. Skin/fat and kidney samples did not have detectable residues after 6 hours withdrawal. No DIA residues were

detected in muscle samples at any time. No antimicrobial activity was detected in any other tissue by the inhibition assay using *Micrococcus luteus* (LOD = 5 µg/kg), indicating that DIA residues detected in liver and kidney were due to microbiologically inactive metabolites of the drug.

In a similar study conducted in turkeys fed a commercial diet containing avilamycin *ad libitum* for 7 consecutive days, residues at zero withdrawal time were 68-195 µg avilamycin equivalents/kg in liver and 37-105 µg avilamycin equivalents/kg in fat/skin and below 28 µg avilamycin equivalents/kg tissue in muscle and kidney. Antimicrobial activity was not found in liver, kidney and muscle samples.

In rabbits fed a commercial diet containing avilamycin *ad libitum* for 7 consecutive days the residues at zero withdrawal time were very low in liver and kidney (93-145 µg avilamycin equivalents/kg and 228-352 µg avilamycin equivalents/kg, respectively, and below 28 µg avilamycin equivalents/kg tissue in muscle and fat. Antimicrobial activity was not found in liver, fat, kidney and muscle samples.

For considering MRLs, an estimate of marker residue (DIA) to total residues was calculated. For pig liver, available data indicate a ratio of 0.5. For the other pig tissues and the other species, this ratio could not be established on an experimental basis owing to the low or non-detectable residue concentrations. A conservative ratio of 0.1 was considered appropriate for recommending MRLs in other species and tissues.

Analytical methods for residues of avilamycin in pig and chicken tissues (muscle, skin/fat, liver and kidney) have been developed. The applicability of the methods to turkey and rabbit tissues was demonstrated to measure DIA-avilamycin equivalents. The DIA concentration was measured by gradient HPLC using negative-ion electrospray ionization mass spectrometry and converted to avilamycin equivalents by multiplying the determined DIA concentration by the molar ratio of avilamycin to DIA (5.6:1). The method was validated by the sponsor at three concentrations for all tissues in all species. The sponsor adopted the LOQs as the minimum concentration in fortified samples shown to satisfy the criteria for recovery and precision, however, this is not always the case.

The Committee reconsidered the data provided and calculated the LOQs considering the representative chromatograms of the extracted fortified samples for pigs and chickens and using the LOQ criterion of signal to noise ratio equal to 10. The LOQs expressed as DIA determined for pigs are 24, 22.4, 3.3 and 10 µg/kg for muscle, skin/fat, kidney and liver, respectively. The LOQs expressed as DIA for chickens are 18.8, 18.7, 22.4 and 30.4 µg/kg for muscle, skin/fat, kidney and liver, respectively.

While the method is satisfactory for measuring avilamycin residues as DIA in a quantitative manner, it requires relatively complex instrumentation that may not be available in all regulatory laboratories. It may be necessary to use alternative methods in these situations.

MAXIMUM RESIDUE LIMITS

The following data have been taken into account in recommending MRLs for avilamycin:

- A toxicological ADI of 0–2 mg/kg bw was established, which is equivalent to a daily intake of 0-120 mg for a 60 kg person.
- Avilamycin is poorly absorbed and extensively metabolized.
- Metabolism studies are available in rats and pigs. No metabolism data are available for chickens, turkeys or rabbits.
- DIA was selected as the marker residue and liver is a suitable target tissue.
- Residue concentrations of the marker residue were not quantifiable or detected in muscle, skin/fat and kidney in pigs and chickens at a withdrawal time of 0 h or greater. Low residue

concentrations were present in liver of all species studied in the first hours post-treatment, but were not quantifiable or detected after 24 h withdrawal.

- For pig liver, a ratio of marker residue to total residue of 0.5 has been established. For the other pig tissues and the other species, the ratio could not be established on an experimental basis owing to the low or non-detectable residue concentrations. A conservative ratio of 0.1 was adopted.
- No microbiologically active residues were detected in edible tissues of pigs, chickens, turkeys or rabbits.
- A validated routine analytical method for the determination of the marker residue in edible tissues of pigs, chickens, turkeys and rabbits is available.
- A conservative estimate of approximately 10 × LOQ expressed as DIA was used to recommend MRLs for chickens. Pig MRLs have been harmonized with chicken MRLs. Chicken MRLs may be extended to turkeys based on similarity between the species. For rabbits, as a minor species, MRLs were harmonized based on the existing recommended MRLs in major species.

The recommended MRLs are expressed as the marker residue, DIA. Rounded MRL values are 200 µg/kg for muscle, 200 µg/kg for skin/fat, 200 µg/kg for kidney and 300 µg/kg for liver for pigs, chickens, turkeys and rabbits.

The EDI was not determined because of insufficient quantifiable data points with which to calculate the median values of residues (low quantities of residues or absence of quantifiable residues).

Using the model diet and the ratio of avilamycin equivalents to DIA, the recommended MRLs would result in a daily intake of 5.3 mg of avilamycin, approximately 4% of the upper bound of the ADI.

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DEXAMETHASONE

First draft prepared by
Bruno LE BIZEC, Nantes, France

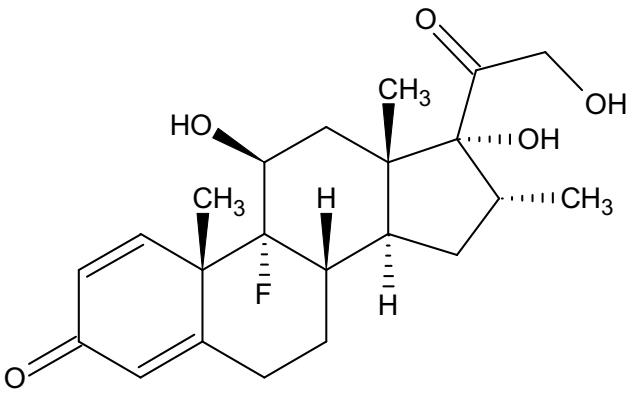
ADDENDUM
to the Dexamethasone monograph prepared by the 42nd , 43rd and 50th meetings of the Committee and published in FAO Food and Nutrition Paper 41/6, 41/7 and 41/11, respectively

IDENTITY

Chemical name: (11β,16α)-9-fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione

Systematic name: (8*S*,9*R*,10*S*,11*S*,13*S*,14*S*,16*R*,17*R*)-9-Fluoro-11,17-dihydroxy-17-(2-hydroxyacetyl)-10,13,16-trimethyl-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3*H*-cyclopenta[*a*]phenanthren-3-one (IUPAC)

Structural formula:



Molecular formula: $C_{22}H_{29}FO_5$

Molecular weight: 392.45

Pure active ingredient: Dexamethasone

INTRODUCTION

Dexamethasone is a fluorinated glucocorticosteroid and a potent anti-inflammatory agent used frequently for treatment of inflammatory processes and primary ketosis in domestic food producing animals. Dexamethasone lacks effects on electrolyte balance but is 30-35 times more potent than cortisol as an anti-inflammatory agent.

Dexamethasone was evaluated at the forty-second and forty-third meetings of the committee (Wells, 1994a,b). At the forty-second meeting, the Committee established an ADI of 0-0.015 µg/kg of body weight for dexamethasone and recommended the following temporary MRLs: 0.5 µg/kg for muscle, 2.5 µg/kg for liver and 0.5 µg/kg for kidney, expressed as parent drug, in cattle and pigs; and 0.3 µg/l for cows' milk, expressed as parent drug. As its forty-second meeting, the Committee noted that dexamethasone undergoes extensive metabolism. However, it also noted that the metabolites did not exhibit any biological activity and consequently proposed dexamethasone as the marker residue. The MRLs were designated as temporary because an adequate method to determine compliance with the

MRL was not available. At the forty-third meeting, the same temporary MRLs were recommended for horses as were recommended at the forty-second meeting for cattle and pigs.
Performance data were requested on the analytical method for evaluation at the forty-eighth meeting of the Committee, but no data were provided. The temporary MRLs for dexamethasone were withdrawn at that meeting due to lack of an adequate analytical method allowing enforcement of the MRLs. At the 50th meeting, the Committee reviewed documentation on an HPLC-MS method (thermospray ionisation, selected ion monitoring) for the control of dexamethasone residues in tissue and milk (Cook and McCormack, 1996; Curl and McCormack, 1996). The chromatograms provided with the report showed some apparent retention time instabilities. Selectivity was not judged adequate because of the partial co-elution of betamethasone (16β-isomer); unambiguous identification of dexamethasone was considered as difficult. Large variation in detector response was reported to occur during analysis. Calculation of quantitative results in incurred samples may not be accurate because non-specific interferences are encountered occasionally. Even if the criteria recommended by the Codex Alimentarius Commission, Volume 3, Residues of Veterinary Drugs in Foods, for accuracy and precision were used and fulfilled, the Committee concluded that the method did not meet the required performance criteria for identification and quantification of incurred residues in tissues and milk.

ANALYTICAL METHODS

Two sponsors provided three methods for the quantification of dexamethasone in muscle/kidney, liver and milk.

Liquid chromatographic methods based on UV detection were considered unsuitable for residue analysis at sub-µg/kg (or sub-µg/l) concentrations. Methods based on GC-MS (negative chemical ionization) after dexamethasone oxidation are no longer in use. Liquid chromatography electrospray (positive or, better, negative mode) mass spectrometry methods are preferred because they provide improved sensitivity and specificity.

The analytical method for muscle and kidney from cattle consists of one common procedure using LC-MS/MS (ESI+). Sample preparation was performed using two solid-phase extraction (SPE) purification steps. The chromatographic method involves gradient elution using a reversed-phase column. No mention of the use of any internal standard was provided for muscle and kidney. The analytical method for liver and milk from cattle consists of two different procedures using LC-MS/MS (ESI−). Sample preparation was performed using one SPE purification step. The chromatographic method involves isocratic elution using a reversed-phase column. A chemical analogue (deltafludrocortisone) was utilized for dexamethasone identification and quantification.

The validation of the method for muscle and kidney was conducted with a target residue level set at 1 µg/kg, whereas the MRL recommended previously by the Committee was 0.5 µg/kg. The validation of the method for liver tissue was performed for a target residue level of 2 µg/kg, whereas the previous MRL in liver was 2.5 µg/kg. The validation of the method for milk was performed at a target residue level of 0.3 µg/l, equal to the previously recommended MRL for milk.

Sample preparation

Muscle, kidney. The sample is denatured in acid and then digested overnight with protease enzyme. After digestion, isopropanol is added to facilitate the extraction of the analytes. The mixture is diluted and passed through a C_{18} cartridge followed by further clean up a SPE anion exchange column and then onto a C18-SPE cartridge. The analytes are eluted from the SPE cartridges, evaporated to dryness, and the dried extracts are reconstituted in mobile phase for further determination by LC-MS/MS.

Milk. Sample preparation is based on protein precipitation using trichloroacetic acid. Clean-up is carried out using solid phase extraction. The final sample solution is analysed by liquid

chromatography (LC) with tandem mass spectrometric (MS/MS) detection using negative electrospray ionisation.

Liver. After enzymatic hydrolysis of the glucocorticosteroid conjugates, the free and aglycone residues are extracted using methanol. The extracts are then centrifuged, evaporated, dissolved in water and cleaned up by SPE. After SPE treatment, the methanol eluates are evaporated and the dry residue is dissolved in the mobile phase. The samples are analysed by liquid chromatography (LC) with tandem mass spectrometry (MS/MS) using negative electrospray ionisation.

Analytical measurement

Muscle, kidney. The chromatographic method (HPLC) was based on gradient elution using a C18 (2 x 150 mm; 4 µm) reversed phase column. The mobile phase consisted of acetonitrile and 50/50 - 0.01% formic acid/0.01M ammonium formate. Flow rate was set at 0.2 ml/min. Injection volume was 20 µl, column temperature was set at 40°C. Total run time was 40 min. The detection of dexamethasone was performed by electrospray (ESI, positive mode) ionisation tandem mass sepctrometry (triple quadrupole mass analyser). Capillary was set 2.5 kV, source temperature at 120°C. Selected Reaction Monitoring was employed and dexamethasone was monitored at 393>373 for quantification, and 393>355, 393>147, 393>337 for identification. No internal standard was used for identification (retention time criteria) nor quantification (calibration curve).

Liver, milk. The chromatographic method (HPLC) was based on an isocratic elution using a Hypercarb C18 column (2.1 x 100 mm; 5µm) reversed phase column. The mobile phase consisted of a mixture of acetonitrile - 0.1% formic acid (90/10; v/v). Flow rate was set at 0.6 ml/min for screening and 0.22 ml/min for confirmation. Injection volume was 20 µl, column was set at room temperature. The detection of dexamethasone was performed by electrospray (ESI, negative mode) ionisation tandem mass spectrometry (triple quadrupole mass analyser). Capillary was set 2.7 kV, source temperature at 120°C. Selected Reaction Monitoring was employed and dexamethasone was monitored at 437>361 for quantification (collision energy 18 eV), plus 437>345 (CE 25 eV) for identification. Deltafludrocortisone (DFUD) was used as an internal standard.

Method validation

The validation was conducted at 1 µg/kg for muscle and kidney, 2 µg/kg for liver and 0.3µg/kg for milk.

Stability

Muscle, kidney. Analytes obtained from tissue extracts are stable over the period of a typical analyses cycle. Analytes are stable under frozen conditions (-20°C) for up to 10 weeks. Standard solutions prepared in methanol using the analytes are stable for up to 1 year.

Liver, milk. The stock standard solutions have been found to be stable for at least 22 months for dexamethasone at –20°C. No information was given for the sample extract.

Specificity and selectivity

Muscle, kidney. The presented method demonstrated its ability to provide non-interfered signals. The technique of acquisition (SRM) used on the triple quadrupole instruments permitted it to eliminate most of the interferences. Betamethasone previously pointed out as a source of potential interfering signal (isobaric compound, close retention time when analysed on reverse phase liquid chromatography) was completely separated from dexamethasone because of the stationary phase. Betamethasone is eluted before dexamethasone and chromatographic peaks are fully separated (e.g. 19.5 min and 20.2 min, respectively). Finally, the method was able to detect truly negative samples uncontaminated with dexamethasone (Boison, et al., 2008).

Liver, milk. The method demonstrated its ability to provide non-interference signals for dexamethasone and its internal standard (i.e. deltafludrocortisone) especially when an HPLC column Hypercard 100 x 2.1 mm is used. The SRM acquisition of the signals provided high specificity. Betamethasone a 16-stereoisomer of dexamethasone was fully chromatographically separated eluting after dexamethasone (e.g. 3.5 min and 4.6 min respectively). Finally, the method was able to detect true negative samples uncontaminated with dexamethasone. Deltafludrocortisone eluted at 2.8 min.

Accuracy-Trueness

Muscle, kidney. Within-day and between day accuracy data generated from the method showed that quantitation can be performed with a trueness below 10% (%RSD).

Liver, milk. Trueness was determined by spiking a pooled sample of bovine liver at 0.5, 1.0 and 1.5 times the MRL for dexamethasone. The matrix matched standard curve was prepared from the same pooled sample as the other spiked samples. Trueness was 15% or better at the three levels either in the screening or confirmatory methods both for milk and liver.

Accuracy-Precision

Muscle, kidney. Within-day and between day accuracy data generated from the method showed that quantitation can be performed with a precision below 10% (%RSD).

Liver. The repeatability (within day) for the confirmation set-up was determined by analysing 6 replicates of a pooled bovine liver sample on 3 occasions spiked at 0.5, 1.0 and 1.5 times MRL. In addition, the repeatability (within day) for the confirmation set-up was determined by analysing a total of 20 different samples on three occasions (spike level at 1 MRL). Repeatability was better than 10.3% (CV) in the pool samples and 12.6% (CV) for the different samples. Reproducibility (within-day and inter-day) was better than 20.4% (CV).

Milk. The repeatability was determined for 20 different samples spiked at the 1-MRL level on three different occasions. Repeatability was better than 10.7% (CV). Reproducibility (within-day and inter-day) was better than 24.6% (CV).

Accuracy-Recovery

Muscle, kidney. The mean absolute recovery was calculated at each of the six calibration points in one of two ways; by either comparing the slope of the calibration curve obtained from the matrix fortified sample to the slope of the chemical standard or matrix matched standard curve, or comparing the interpolated concentrations at each of the six calibration points, pooling them together and calculating the mean absolute recovery over the calibration range. Dexamethasone mean recovery was 66 ± 4 %.

Liver, milk. The absolute recoveries were determined by comparing the absolute peak area response for six individual blank samples spiked at the MRL before sample preparation with the same six blank samples spiked after sample preparation. In this case no internal standard was used, and quantification was carried out using external absolute response. When diluting the extracts, care was taken to ensure that the suppression/enhancement effects were the same in samples spiked before and samples spiked after the extraction. In liver recovery values were 70.0 ± 10.6 % for the screening method and 69.0 ± 15.5 % for the confirmation process. For milk, lower values have been found, i.e. below 25% for dexamethasone.

Limit of detection, quantitation, decision limit, detection capability

Muscle, kidney. Linear regression analysis was performed on three sets of calibration standards (i.e., chemical standards, matrix matched standards, and matrix fortified standards). Calibration curves from a minimum of three different days were pooled and analysed. Calibration curves generated from the chemical standards were linear over the calibration range of 0.5 – 10.0 ng g^{-1} with a correlation coefficient better than 0.996. Similarly, calibration curves generated from matrix fortified and matrix matched standards were also linear over the same analytical range and had a correlation coefficient better than 0.992. The claimed limits of quantification and identification were both 0.4 µg/kg. Limit of decision (CCα, risk α=5%) and detection capability (CCβ, risk β=5%) were 1.2 µg/kg and 1.5 µg/kg. The validation has been conducted with a MRL set at 1 µg/kg wheras the recommended MRL by the JECFA was 0.5 µg/kg (the EMEA fixed the MRL at 0.75 µg/kg).

Milk. The calibration curve was tested in the range 0-0.6 µg/L. The correlation coefficient (R^2) was found to be better than 0.95. The claimed limit of quantification was defined as the lowest validated level which was 0.15 µg/L. Limit of decision (CCα) was 0.45 µg/L. Detection capability (CCβ) was 0.57 µg/l.

Liver. The calibration curve in bovine liver was tested in the range of 0-4 µg/kg. The correlation coefficient (R^2) was found to be better than 0.95. The claimed limit of quantification was defined as the lowest validated level which was 1µg/kg. Limit of decision (CCα) was 2.9 µg/kg. Detection capability (CCβ) was 3.7 µg/kg.

APPRAISAL

General

Muscle, kidney, liver, milk. Liquid chromatographic methods based on UV detection were considered unsuitable for residue analysis at sub µg/kg (or l^{-1}) concentrations. Methods based on GC-MS (negative chemical ionisation) after dexamethasone oxidation are no longer used. Liquid chromatography electrospray (positive or better negative mode) mass spectrometry methods were developed, validated and used. This technology is now available is most laboratories worldwide.

Analytical method

Muscle, kidney, liver, milk. Tissue sample preparation was performed using at least one (milk, liver) or two (muscle, kidney) SPE purification steps. The chromatographic method involves gradient (liver, kidney) or isocratic (milk, liver) elution using a reversed phase column. Electrospray ionisation was used to produce ions further characterised by selective reaction monitoring. No mention regarding the use of any internal standard is provided for muscle and kidney, whereas a chemical analogue (deltafludrocortisone) was utilised for dexamethasone identification and quantification in liver and milk samples.

Method validation

Muscle, kidney, liver, milk. Stability of the target analyte was demonstrated in standard solution (all matrices), and biological extract (muscle, kidney). Selectivity was proved as fitting with the needs, especially the efficient chromatographic separation of betamethasone and dexamethasone was made possible. The validation for muscle and kidney was conducted with a target residue level of 1µg/kg wheras the MRL recommended previously by the Committee was 0.5 µg/kg. The validation for liver has been performed with a target residue level of 2 µg/kg wheras the MRL recommended previously by the Committee was 2.5 µg/kg. The validation for milk was performed with a target residue level of 0.3 µg/l equal to the previously recommended MRL for milk. The claimed limits of quantification and identification were 0.4 µg/kg both for muscle and kidney. The claimed limits of quantification were 0.15 µg/kg for milk and 1 µg/kg for liver (LOQ defined as lowest validated level).

Limit of decision (CCα, risk α=5%) and detection capability (CCβ, risk β=5%) were 1.2 µg/kg and 1.5 µg/kg for muscle and kidney. These performance values fit the expectations of a MRL method at 1.0 µg/kg, but are insufficient considering the MRL as recommended by the Committee at its forty-second and forty-third meetings.

Limits of decision and detection capabilities were 0.45 µg/L and 0.57 µg/l for milk (MRL recommended by JECFA at 0.3 µg/l), and 2.9 µg/kg and 3.4 µg/kg for liver (recommended MRL by JECFA at 2.0 µg/kg). These performance values have been calculated in reproducibility conditions, which can be considered as conservative. Any laboratory implementing the methods will characterize an "in-house" decision limit in repeatability conditions. The CCα would then be significantly closer to the MRL. The same applies to the CCβ. In summary, the performances of the methods fulfil the minimum performance criteria corresponding to dexamethasone residues in milk and liver at the MRL as recommended by the Committee in its forty-second and forty-third meetings.

Conclusion

A suitable validated routine method was available for monitoring dexamethasone in bovine milk and liver at 0.3 µg/l and 2.0 µg/kg, respectively. A suitable validated routine method was available for monitoring dexamethasone in bovine muscle and kidney at 1.0 µg/kg, but not at 0.5 µg/kg. No validated method for horses and pigs was provided or could be found, but the method provided for cattle tissue is adequate to be extended to pig and horse tissues.

MAXIMUM RESIDUE LIMITS

In recommending MRLs for dexamethasone, the Committee considered the following factors:

- The established ADI is 0–0.015 µg/kg-bw, equivalent to 0–0.9 µg for a 60-kg person.
- The marker residue is dexamethasone.
- The appropriate target tissues are liver or kidney and milk.
- A suitable validated routine method was available for monitoring dexamethasone in bovine milk and liver at 0.3 µg/l and 2.0 µg/kg, respectively.
- A suitable validated routine method was available for monitoring dexamethasone in bovine muscle and kidney at 1.0 µg/kg, but not at 0.5 µg/kg.
- No validated method for horses and pigs was provided or could be found, but the method provided for cattle tissue is adequate to be extended to pig and horse tissues.
- The recommended MRLs are based on the performances of the analytical methods at twice the LOQ.

On the basis of the above considerations, the Committee recommended the following MRLs for edible tissues of cattle, pigs and horses, expressed as the marker residue dexamethasone: muscle/kidney, 1.0 µg/kg; liver, 2.0 µg/kg; cow's milk, 0.3 µg/l. Based on these values for the MRLs, the maximum theoretical intake would be 1µg/day per person. This would be compatible with a maximum ADI of 0.9 µg for a 60-kg person. The Committee noted that at its forty-second meeting it was concluded that dexamethasone is rapidly eliminated from muscle and milk, and that the probability of exposure to residues from these tissues is low.

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MALACHITE GREEN

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IDENTITY AND PROPERTIES

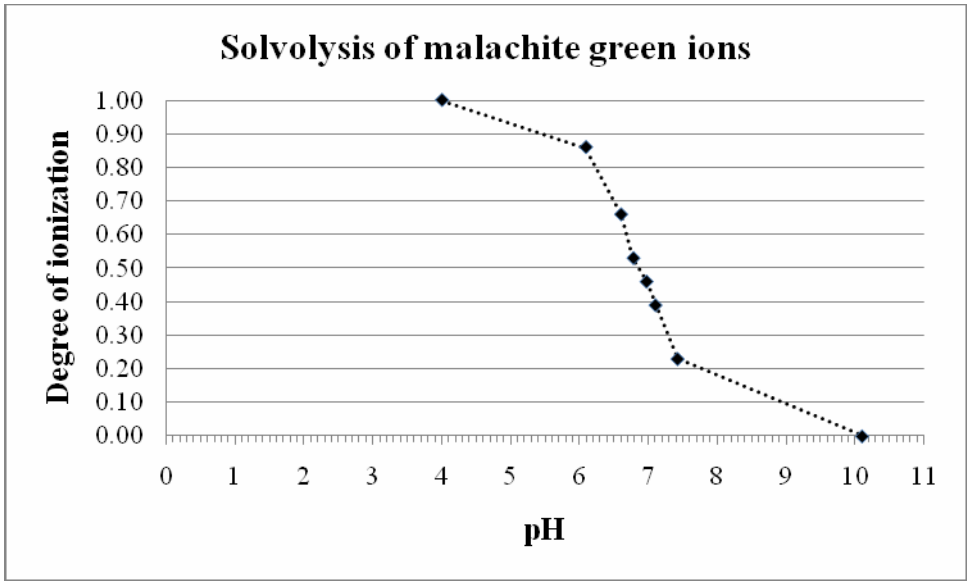
The structural identity, some major physical-chemical properties and molecular characteristics of malachite green salts, its carbinol base and its major metabolite leucomalachite green are summarised in Table 1. Other important properties are briefly summarised in the below subsections.

Chemical properties

Solvolysis

Goldacre and Phillips (1949) investigated the solvolytic reaction of malachite green and the formation of the carbinol at various pH values. For 2.7 x 10^{-4} M solutions at 25°C they found the following degrees of ionization shown in figure 1.

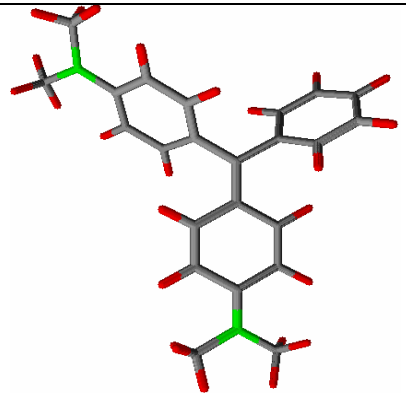
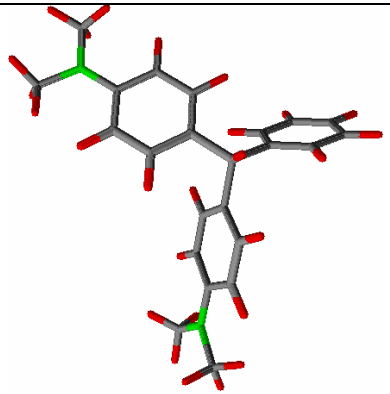
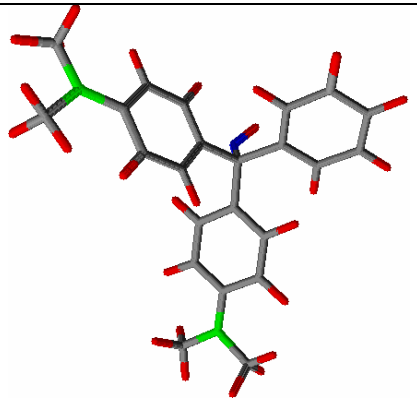
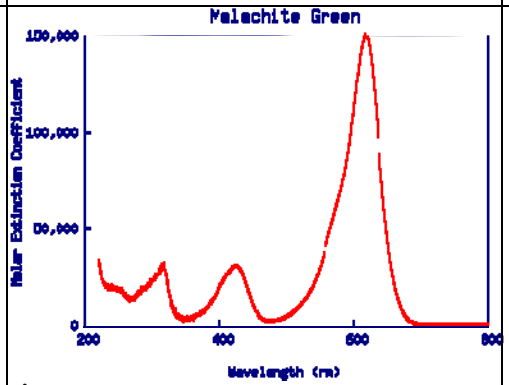
Figure 1: Solvolysis of malachite green as function of pH



From these results a pK of 6.9 was calculated. At this pH the time to decline half-way from 100% ionization to the equilibrium value is 2.1 hours. Velocity constants for the solvolytic reaction increase with increasing pH. The carbinol base which is less soluble than the ionized form is the form in which malachite green is probably taken up by fish. Therefore, the solvolytic equilibrium influences the pharmacokinetics of bath treatments.

Table 1: Physical-chemical properties of malachite green, its carbinol base and its metabolite leucomalachite green.

Substance name	Malachite green	Leucomalachite green	Malachite green carbinol base
Depositor-supplied synonyms (PubChem)	Total number: 82 Examples: Methylene green Grenoble Green Victoria Green Aniline Green Benzal Green China Green Fast Green Burma Green B Diamond Green Bx	Total number: 38 Examples: Leuco malachite green Malachite green leuco Malachite green leuco base Tetramethyldiaminotriphenylmethane C.I. Basic Green 4, leuco base	Total number: 20 Examples: Solvent Green- 1 Malachite Green carbinol Malachite Green Carbinol base
Chemical Abstracts Registry number	10309-95-2 (chloride: 569-64-2) (oxalate: 2437-29-8)	129-73-7	510-13-4
PubChem-CID	11295	67215	10521
EINECS	209-322-8 (chloride) 219-441-7 (oxalate)		208-109-7
IUPAC	[4-[(4-dimethylaminophenyl)-phenylmethylidene]-1-cyclohexa-2,5-dienylidene]-dimethylazanium	4-[(4-dimethylaminophenyl)-phenylmethyl]-N,N-dimethylaniline	bis(4-dimethylaminophenyl)-phenylmethanol
Molecular formula	$C_{23}H_{25}N_2$+	$C_{23}H_{26}N_2$	$C_{23}H_{26}N_2O$
Formula weight	329.46	330.47	346.47

Structure (propeller-like) prepared with Chemsketch 10.0			
UV-VIS spectrum	 λ_{max} is 616.5 nm H. Du, R. A. Fuh, J. Li, A. Corkan, J. S. Lindsey. PhotochemCAD: A computer-aided design and research tool in photochemistry. *Photochemistry and Photobiology,* 68, 141-142, 1998.	Not found	Not found
IR spectrum	Not found	http://webbook.nist.gov/cgi/cbook.cgi?ID=C129737&Units=SI&Mask=80#IR-Spec	Not found
Melting point [°C]	164	About 100	112.0 - 114.0

	(MG oxalate; Mallinckrodt-Baker 2006, http://www.jtbaker.com/msds/englishhtml/m0286.htm)		(Sigma-Aldrich 2006)
Octanol/water partition coefficient: $\log K_{ow}$	0.62 (MG chloride)	Not found	Not found
Solubility in Water	40 g/l at 25°C (MG chloride) Baughman, G.L., et al; Dye Solubilities in: Advances in Color Chemistry, Freeman, M., Peters, M.T., eds., NY, NY: Elsevier (1993)	No information available	Low, exact data not found
Impurities found in commercial products[1]	Leucomalachite green, carbinol and demethylated derivatives and 4-DMABP	Malachite green and mono-desmethyl leucomalachite green	

Malachite green is typically available as hydrochloride or oxalate salt. The hydrochloride may have been obtained through precipitation with Zinc chloride and may be highly toxic to fish.

[1]See for example LeGoff and Wood (2008)

Other physical-chemical properties

Adsorption

Adsorption characteristics of malachite green were studied in order to find ways to remove it from waste waters. Traditional methods of wastewater cleaning only partly remove synthetic dyes such as, malachite green and structurally related compounds. The adsorption on a variety of inexpensive and more or less efficient inorganic and organic solid supports was tested as a method for the removal of malachite green from water and wastewater. When testing the adsorption characteristics of malachite green from aqueous solutions frequently only the kinetics of decolourisation of the aqueous solution was measured. Garg, et al. (2003) studied the properties of chemically treated saw dusts (e.g., formaldehyde, sulphuric acid). Gong, et al. (2006) studied citric acid modified rice straw and soybean hulls esterified with phosphoric acid. Hameed and El-Khaiary (2008) used rice straw-derived char. Mittal (2006) determined the adsorption kinetics from waste water onto hen feathers. Janos, et al. (2005) studied the sorption of malachite green from waters onto a naturally occurring kind of weathered and oxidized young brown, acid stable coal called oxihumolite. Tahir and Rauf (2006) used bentonite clay for the removal of malachite green from aqueous solutions. Wang and Ariyanto (2007) studied adsorption of malachite green to natural zeoliths.

Carbon-based sorbents show excellent adsorption properties for a considerable number of synthetic dyes. The preparation of carbon sorbents is generally energy consuming and large amount of carbon sorbent is needed for the removal of dyes from large volumes of water. Consequently, according to Forgacs, et al. (2004), the use of commercially available products is fairly expensive. Activated carbon can remove malachite green from water. A system for removal of malachite green from waters used for antifungal treatment in hatcheries was developed and described by Marking, et al. (1989). The removal efficiency was significantly less than 100%. For solutions containing 2 mg/l of malachite green an average of 23.4 mg of malachite green were adsorbed per gram of carbon.

Recent studies on the sorption using activated carbon with BET surface areas in the order of 1000 m^2/g prepared from different sources of material using various models for the calculation of adsorption isotherms (linearised and non-linear) and for studying the kinetics of adsorption. According to some authors the adsorption of malachite green was best described using a pseudo-second-order model with intra-particle diffusion of malachite green molecules within the carbon particles as a rate-limiting step. The following materials (examples only) were used for the preparation of activated carbon:

Authors	Material used
Singh and Rastogi (2004)	Used tea leaves
Rahman, et al. (2005)	Rice husks
Başar (2006)	Waste apricot
Onal (2006)	Waste apricot
Onal, et al. (2006, 2007)	Lignite,
Malik, et al. (2007)	Groundnut shell waste.
Kumar (2006), Kumar and Sivanesan (2006)	No information on source material
Porkodi and Kumar (2007)	Jute fiber.
Zhang, et al. (2008)	Arundo donax root

Several groups determined thermodynamic parameters (ΔH, ΔG, and ΔS) and concluded that the reaction was endothermic.

Malachite green is markedly biosorbed by activated sludge and reduces the rate of oxygen uptake by activated sludge proportionally to its biosorption (Mihara, et al., 2005). The results of the above studies suggest that malachite green and its metabolites and breakdown products may not be

completely removed by wastewater treatment and may be present in sufficient amounts in effluents from industry or waste water treatment plants or other sources to cause residues in wild fish. A group of researchers has recently claimed – without providing convincing evidence - that they have published the first example where malachite green was demonstrably taken up by eels caught downstream from treatment plants in the lakes and rivers surrounding Berlin, Germany (Schuetze, et al., 2008).

Photodegradation

Hydrogen peroxide has been frequently applied to decolorize synthetic dyes including malachite green in waters in the presence of suitable photo catalysts. For example, micro porous solid material prepared by precipitation of phosphotungstic acid and potassium ions, followed by calcinations was proposed for this purpose (Chen, et al., 2006). The formation of active oxygen species such as the radicals O_2^-, HO_2 and OH are detected during the degradation of dye, and they are proposed to be responsible for the degradation of dyes. In such systems CO_2 and small organic acids are the main reaction products.

The presence of catalysts such as TiO_2 enhances the rate of photodecomposition of malachite green under visible light. Both the superoxide anionic radical and the dye cationic radical are essential to the mineralization of the dyes under visible light-induced photo catalytic conditions (Arpaç, et al., 2007). Kominami, et al. (2003) used TiO_2 nano-particles with various physical properties that had been prepared by hydrothermal crystallization in organic media (HyCOM) and post-calcination, for photo catalytic decomposition of malachite green in an aqueous suspension under aerated conditions. Adsorptivity is a decisive factor for the initial bleaching of malachite green on this material which follows pseudo zero-order kinetics. Chen, et al. (2007) have studied the reaction mechanism of malachite green photo degradation on TiO_2. They identified a series of N-demethylated intermediates (mono-, di-, tri-, and tetra-demethylated malchite green) under basic reaction conditions of the process. These degradation products are also known to be formed metabolically in bacteria and animals (see below). Under acidic conditions, the whole conjugated chromophore structure of malachite green was cleaved (Chen, et al., 2007). Hydrogen peroxide can effectively decolorize dye wastewaters in the presence of Fe(III) – loaded ion exchange resin (Amberlite IRA 200). The degradation process of Malachite green proceeds via demethylation and phenyl ring openings before CO_2 and small molecules are formed.

Binding to macromolecules

Malachite green can bind to macromolecules. Of interest is the binding to small artificial RNA molecules (aptamers). The complex can exhibit interesting new properties, for example enzymatic activities (Brackett and Dieckmann, 2006). In the binding process the RNA adapts to the ligand (“adaptive binding”), but the ligand itself also undergoes conformational changes ("induced fit") (Nguyen, et al., 2002; Nguyen et al., 2004). The crystal structure of such complexes has been studied using tetramethylrosamine (TMR), a high-affinity analogue of malachite green (Baugh, et al., 2000). The properties and a number of applications of such complexes have been published. One possible use is the determination of malachite green itself, because aptamers are known which enhance malachite green fluorescence by factors in the order > 1000.

It is long known that malachite green binds to DNA (Nordén, et al., 1978; Bhasikuttan, et al., 2007). Cationic triarylmethane dyes also have complex-forming properties with proteins (Tacal and Ozer, 2004). A full discussion of all these properties is beyond the scope of this monograph.

Industrial uses of malachite green

Malachite green is used extensively as a dye for leather, wool, cotton, jute, paper, certain fibres, etc. For such purposes it has been produced in large quantities and extremely variable qualities. About 10-

15% of all dyes are directly lost to wastewater in the dyeing process (Parshetti, et al., 2006). Frequently the purity of products used in biological studies has not been reported. In human medicine the carbinol is/has been used as a wound antiseptic and as a treatment of mycotic skin infections, and in staining of tissues and bacteria.

RESIDUES IN FOOD FROM AQUATIC SPECIES AND THEIR EVALUATION

Conditions of use in aquatic animals

Malachite green has been used as a fish fungicide in closed systems alone or in combination with other chemicals such as formaldehyde for decades. Frequently reported concentrations are about 0.05 - 0.1 mg/kg. It is important to use zinc free preparations in order to avoid metal intoxications of the fish. Therefore, the oxalate was most frequently used. Foster and Woodbury (1936) were reportedly the first to introduce its use as fungicide and antiseptic. It took almost 47 years until researchers considered for the first time the possibility that malachite green could be taken up by fish (Poe and Wilson, 1983).

Malachite green has been used for the treatment of eggs of fish and crayfish. Malachite green is also an effective topical and systemic antiprotozoal agent. Reported types of treatment of fish include dip treatment, flush treatment, sustained culture treatment and application in feed. Extremely wide ranges of concentrations and exposure times have been used (review by Alderman 1985).

In a review of historical uses of malachite green, Sudova, et al. (2007) discriminate between dip treatments of 10-30 seconds duration and concentrations up to 100 mg/L to treat topical fungal infections, short-term malachite green bath treatments of 60-90 minutes duration at 6.7 mg/L, and long-term bath treatments of six days duration at 0.15 mg/L for salmonids and 0.5 mg/l for cyprinids. They state that this type of treatment was used to control protozoan ectoparasites, particularly the ciliated protozoan *Ichthyophthirius multifilii*s. Malachite green concentrations can be reduced in multi-component baths, for example in combination with formaldehyde. Treatment with malachite green can produce numerous side-effects in treated fish and fish eggs.

An important factor determining therapeutic and toxic effects is the temperature. Batch to batch variation in concentration and purity of the dye and lack of standardization of test conditions have been major confounding factors in the judgment of effectiveness of doses and exposure times. Therefore, it goes beyond the scope of this monograph to make any conclusive statements and comparisons about dosages and other conditions of use. Information on treatment conditions will be given individually in connection with the discussion of pharmacokinetic and residue studies.

Malachite green is toxic to fish, in particular to small fry. Bills, et al. (1977) used standard laboratory tests in order to determine the LC_{50} under various conditions of temperature, pH, and hardness of the treatment bath and of duration of treatment. Fingerling fish of a great variety of species, weighing 0.5 to 1.5g were used for the tests. Increase in exposure time significantly increased the acute toxicity. In short term-exposure (3 and 6 hours) of rainbow trout and channel catfish higher temperatures increased the acute toxicity. At the longest tested exposure time (96 hours) the temperature effect disappeared in rainbow trout. pH and hardness had no significant influence on acute toxicity. As an example, some data obtained with rainbow trout were selected from the original paper and are presented below in Table 2. The original work also provides the 95% conficence intervals of the LC_{50} which is not given in the below Table 2.

Table 2: Acute toxicity of malachite green to rainbow trout

Temperature [°C]	Water hardness	pH	Incubation time [hours]			
			3	6	24	96
			LC_{50} [mg/L			
7	soft	7.5	>2	2.3	0.4	0.17
12	soft	6.5	>2	1.0	0.28	0.28
12	soft	7.5	1.4	0.8	0.36	0.25
12	very soft	8	2.0	0.8	0.36	0.29
12	soft	8	2.3	0.8	0.28	0.23
12	hard	8	2.3	1.4	0.35	0.29
12	very hard	8	2.4	0.8	0.28	0.25
12	soft	8.5	2.6	1.0	0.28	0.21
12	soft	9.5	>2	1.3	0.37	0.17
17	soft	7.5	1.4	0.6	0.57	0.28

PHARMACOKINETICS AND METABOLISM

Pharmacokinetics in Fish

Physiological facts: Relationship between carcass weight and organ weight of fish

Some basic physiological facts are necessary to understand the kinetic behavior of malachite green in fish. Schmelzing and Claus (1990) found that in rainbow trout (*Oncorhynchus mykiss*) absolute organ weights increased with increasing carcass weight while their weights as a proportion of carcass weight decreased. Heart and liver weight were highly correlated with carcass weight, while the correlation between spleen and carcass weight was moderate. According to Corti (1948), muscle meat, liver and kidney make up 70.2, 1.2, and 0.77% of body weight in rainbow trout. The range of body weights (n=7) was 54.1 to 82.3g and corresponding range of muscle meat weight ranged from 61% to 78% of the body weights of the animals. The corresponding figures for eel were 80.9, 1.32, and 0.75 % for muscle meat, liver and kidney, respectively.

Fish physiology: Uptake of hydrophobic compounds

Fish normally take up hydrophobic compounds via the gills by passive diffusion (Gobas, et al., 1986). Rates of uptake can be a function of: water flow over the gills, blood flow through the gills, diffusion through the aqueous stagnant layer along the gill epithelium, or diffusion through the gill membrane. Hayton and Barron (1990) have summarized: "In general, for any particular chemical, only one of the barriers is operative with the resistance offered by the others being negligible. The rate-limiting barrier is determined by the physico- and biochemical properties of the substance: molecular size, lipophilicity, binding to blood proteins and formed elements. The resistance of each barrier is affected differently by variables such as temperature, molecular size, lipophilicity and body size of the animal. When the resistance offered by the gill barriers is low, uptake may be controlled by transfer to storage tissues, e.g., by blood flow to adipose tissue".

When fish increase their water flow, e.g., with decreasing oxygen concentrations or other types of stress, uptake per unit time frequently increases. Sijm, et al. (1994) studied the influence of blood and water flows on the uptake of some hydrophobic compounds by rainbow trout. The fish used in their experiments had an average weight of 110 ± 12g (n not given). The temperature was 12°C. For all compounds studied the uptake rate constants increased with water flow between 0.045 and 0.52 l/min/kg body weight and remained constant at higher flow. The uptake rate constant was constant for blood flow between 4.4 and 10 ml/min/kg body weight but doubled when the blood flow was

increased from 10 to 20 ml/min/kg body weight. From their findings they deduce that water flow will practically limit uptake of hydrophobic chemicals in fish weighing more than 5g.

Gill physiology

In a study on water flow and gas exchange at the gills of rainbow trout (*Salmo Gairdneri*) Erickson and McKim (1990) developed a simple flow limited model for the exchange of organic chemicals at fish gills. The mathematical model for the exchange of organic chemicals by fish gills was formulated based solely on the limitations imposed by the flows of water and blood into the gills. The model could be useful for approximate assessments of accumulation of organic chemicals by fish. For large rainbow trout, the model was found to closely follow the magnitude and trends of observed gill uptake rates over a range of octanol/water partition coefficient from 1 to 10^6 .

Davis and Cameron (1971) estimated the volume of water passing over the gills per unit time (ventilation volume). The technique was direct measurement. For this purpose a rubber membrane was stitched round the margin of the mouth of the fish in a way that it separated inspired and expired water. 18 fish of a body weight of 210.3 ± 2.3 g were used at 8.6°C to determine ventilation volume when the animals were quiet. The estimation was repeated 4-11 times with each animal. The lowest individual estimate of ventilation volume observed in one fish was 22.0 ml/min; the average per fish ranged from 26.0 to 49.0 ml/min. The overall average was 37.0 ±7.4 ml/min. The corresponding mean ventilation rate was 74 breaths/min and the mean ventilator stroke volume was 0.5 ml/breath. When the fish struggled or were disturbed maximum values rose as high as 162 ml/min in one animal (average 88.2 ± 43.7).

Nichols, et al. (2004) developed a physiologically based toxico-kinetic model for dietary uptake of hydrophobic organic compounds. Malachite green administered via the diet shows unsatisfactory efficacy. Therefore, this model cannot be applied to the data discussed in this monograph.

Pharmacokinetic studies in rainbow trout

Alderman and Clifton-Hadley (1993) conducted a pharmacokinetic study in rainbow trout (*Onkorhynchus mykiss*). The dye was administered through uptake from the water bath. The heavily vascularized gill was assumed to be the principal site of malachite green uptake from solution under these conditions

The fish used in the main pharmacokinetic experiments of the study were two separate groups of rainbow trout with average body weights of 241 ± 33 g (n not given) for studies conducted at 16°C and 199 ± 20g (n not given) used for studies conducted at 8°C, respectively. A third group of 30 fish of 50g body weight was used for additional experiments in which residues in muscle were determined in individual fish at 16°C (results not given). The pH was 7.6 and total hardness was 13.8° dH. Under these conditions more than 95% of the malachite green was in the carbinol form. The purity of the malachite green was tested by thin layer chromatography. Treatment solutions containing 1.6 mg/l (4.86 µmoles/l) were prepared from a commercial liquid formulation 15 h before use in order that the dye-carbinol equilibrium concentrations could become established. Treatment time was 40 minutes.

In the main experiments fluids and tissues of five fish per time point were pooled and stored frozen for analysis. However, one graph of the publication shows individual kinetic data in serum obtained in a separate experiment. Samples of serum and bile were allowed to defrost before analysis. After dilution with buffer at pH 4.0 they were extracted for 24 h into pentan-1-ol (no information on the partitioning of malachite green and its metabolites was provided). After 24 h, samples were centrifuged at 2000 × g for 30 min, giving a clear pentan-1-ol supernatant. All other tissues were allowed to defrost overnight and weighted composites were then blended in 2% pepsin adjusted to pH 2.0 with HCl. The blended samples were kept for 18h at room temperature (20°C) and were shaken thoroughly several times in that time. The samples were then partitioned at room temperature at approximately pH 4 into pentan-1-ol. Following the addition of pentan-1-ol the flasks were shaken

vigorously before being left (with further shaking) for a further 24 h. Samples were then shaken thoroughly again before centrifugation at 2000 × g for 30 min at 4°C.

Sample extracts were scanned at wave lengths from 540 to 700 nm. Peak absorption for the malachite green dye ion in extracts was 625 nm. Reported recoveries at 10 mg/kg were 19, 82, 75, 68, and 60% for serum, liver, kidney, muscle, and viscera, respectively. The spectrophotometer was calibrated against extracts of malachite green obtained from representative fish tissues spiked with known concentrations of malachite green and after equilibration subjected to the same extraction procedure as described above for the experimental samples. The reported LOD was about 50 µg/kg (data not shown). Some tissues presented general or occasional problems in residue determination including interference of colored co-extracted substances. The paper exhibits a number of weaknesses:

- Concentration of the drug in water was not monitored during the experiment.
- The description of the experiment lacks precision. The exact relationship between total weight of treated fish and weight of the bath is not given. One may speculate that it not exceeded 24 x 5 x 0.241 kg of fish in 725 kg bath.
- Fat was not sampled.
- It was not determined to what extent leucomalachite green was extracted and whether it was re-oxidized to the malachite green. Therefore, the kinetic data of this study can probably not be interpreted.
- Insufficient information on method validation is provided. The study typically does not provide information on results obtained with individual animals; thus no estimate of the biological variability is possible.
- The authors report that malachite green appeared in the serum very rapidly, with concentrations increasing steadily until the fish were removed from the dye (results not shown). In fact, peak concentrations shown in figure 2 of the paper were in the order of 13-13.5 mg/kg at both 8 and 16°C.
- The text states that peak concentrations in muscle were reached 90-120 min after the end of exposure and gives values of 6.81 and 10.79 mg/kg for the peak concentrations reached at 8°C and 16°C, respectively; however, the curve describing the influence of exposure time never exceeds approximately 1 mg/kg for muscle.
- The text states that when a group of small rainbow trout was exposed to malachite green and individual muscle residues examined at 24 h post-exposure, considerable fish-to-fish variations were evident; however neither data nor an estimate of the variance are provided.
- The legend to the figure describing the kinetics in bile and the corresponding text state that bile could only be reliably collected for the first 40 h; however, the corresponding graph shows data points for 48 and 72 hours.
- The analytical method used was inadequate.
- The calculated half-lives for serum and tissues cannot be compared because they are either based on different kinetic models used for curve fitting, or - even when the same model was used - the distribution in time of the data points covered different phases of the kinetics.
- Some variations in treatment parameters and associated effects on results are discussed without providing any data.
- Extrapolations (calculations and results not shown) far beyond the experimental time points and orders of magnitude below the measured concentrations.

The following conclusions may be drawn from the results of the study: Uptake was lower at 8°C than at 16°C; the initial rate of decrease of the optical density at 625 nm was higher at 16°C than at 8°C; the maximum concentrations found in tissues were in the order of (all values in mg/kg):

Temperature/Tissue	Serum	Liver	Kidney	Muscle
8 °C	13		8	7.8
16 °C	13.5	16.5	34	10.8

However, this statement is only valid if one can assume that the biotransformation of malachite green in this experiment was a slow process compared to the rate of uptake or that leucomalachite green is not picked up by the analytical method. If one assumes that malachite green was rapidly metabolized to leucomalachite green and other molecules (as shown in other studies) which are all extracted, then all results of this study could be meaningless numbers; if leucomalachite green was re-oxidized the above given maximum concentrations could represent an estimate of total residue.

A table summarizing pharmacokinetic and residue data in aquatic species was found on the website of the U.S. FDA. The table includes the work of Alborali, et al., published under the title: "The persistence of malachite green in the edible tissue of rainbow trout (*Oncorhynchus mykiss*)" in the Journal Rivista Italiana di Acquacoltura 32, 45-60. The summaries of the cited findings are given here: Rainbow trout (*Oncorhynchus mykiss*) with a body weight of 60-80 g were exposed at 18 °C for one hour to a solution of 1 mg/l of malachite green. Residues were determined by HPLC (no details given). The following information contained in the FDA document is provided for gills, kidney, muscle and skin:

"Gill: Residues decreased fairly rapidly during the first 320 days reaching 260 by d41, and below 1 ng/g after 7th month. T 1/2 in the 20 d range initially, with later slow decline in the 50d range.
Kidney: Max residue on d(ay) 30 = 1650 ng/g, declined to below 1 ng/g after 41 d.
Muscle*:* Residues high for 34 days - above 1,000 ng/g. Decreased to 200 ng at the 4th month, around 100 by 150 d, slowly declining to below 10 ng/g by 9th month. $T_{1/2}$ in the 40-50 d range.
Skin: Residues decreased fairly rapidly during first 20 days, to about 2500 ng/g, d 50= approx 1000, 200 ng/g round the 4th month, below 1 ng/g at day 283. $T_{1/2}$ in the 50 d range."

Pharmacokinetic studies in channel catfish

The pharmacokinetics and metabolism of malachite green in channel catfish (*Ictalurus punctatus*) were examined by Plakas, et al. (1996) after intravascular dosing or waterborne exposure. The intravascular dosing solution contained 0.8 mg of ^{14}C labeled dye cation per ml of 0.85% aqueous NaCl solution corresponding to a specific activity of 0.925 MBq/ml or 1.16 MBq/mg. For waterborne exposures, the initial dye concentration was 0.8 mg/l corresponding to a specific activity of 0.185 MBq/ml or 0.231 MBq/mg. The channel catfish were 0.5 to 0.7 kg. For the collection of blood and urine the dorsal aorta and urinary bladder were cannulated.

- Five animals were dosed intravascularly with [^{14}C] malachite green at a dosage of 0.8 mg/kg body weight. Blood specimens were collected 2.5, 5, 7.5, 10, 15, 20, 30, and 45 min and 1, 2, 4, 6, 8, and 10 h after drug administration.
- Five animals were transferred to the dosing solution (0.8 mg/l). Blood specimens were taken at 15-min intervals during the 1-h exposure period. At the end of the dosing period, fish were briefly rinsed in a water bath. Blood specimens were taken at 10, 20, 30, and 45 min and at 1, 2, 4, 6, 8, and 10 h after the end of the dosing period.
- To determine the tissue distribution of malachite green and its residues after waterborne exposure, groups of five animals were exposed to [^{14}C]-malachite green solutions (0.8 mg/l) for 1 hour. Animals were killed and tissues collected immediately after dosing (designated 0 h) and at 2, 4, 24, 96, 168, and 336 h (14 days). Additional animals dosed with unlabelled malachite green were sacrificed after 28 and 42 days.

For HPLC determination of malachite green and leucomalachite green, plasma was extracted with acetonitrile. Muscle was subjected to a more complex procedure involving extraction with acetonitrile – acetate buffer, re-extraction, solvent partition, and SPE. HPLC fractions were subjected to post-column oxidation to the malachite green ion. Mean extraction efficiencies for malachite green and leucomalachite green from plasma and muscle were 85 - 95%. Mean recovery of total radioactivity from muscle of treated fish was 88%, however, individual animal data were not provided.

The mean of the concentrations of the plasma samples of five animals declined rapidly after intravascular dosing. Simultaneously the corresponding concentrations of leucomalachite green increased rapidly and reached an average maximum concentration of 0.875 µg/ml in the samples taken at 0.75 h after dosing. At this time point the corresponding concentration of the parent malachite green was 0.6 µg/ml. At ten hours the concentrations of leucomalachite green and of parent malachite green were 0.20 and 0.05 µg/ml, respectively. The sum of these two compounds accounted for approximately 70% of the total drug equivalents at each sampling time. The authors fitted a six parameter (three exponential terms) equation to the data obtained at 14 time points of which nine points were collected during the first hour after treatment. They estimated a terminal half life of 6.2 hours for malachite green.

During waterborne exposure at 21 ^{o}C total radioactivity and the concentrations of both malachite green and leucomalachite green increased very rapidly to 2.77 and 1.56 µg/ml plasma, respectively. Concentrations of malachite green then started declining immediately; however the peak concentration of leucomalachite green was 2.36 µg/ml 1 hour after transfer of the fish to clean water. The authors state that the decline followed a tri-exponential curve (results not shown) and estimated a terminal half life of 4.7 h. After 10 h concentrations of malachite green in plasma had declined to the limit of detection of 0.25 µg/ml. The authors state that the concentrations of leucomalachite green were still 30 times higher. The half life of terminal depletion of leucomalachite green was not estimated. No information on biological variability is available. The water bath conditions were such that the ratio of the ionic and the carbinol form of malachite green was 6:10.

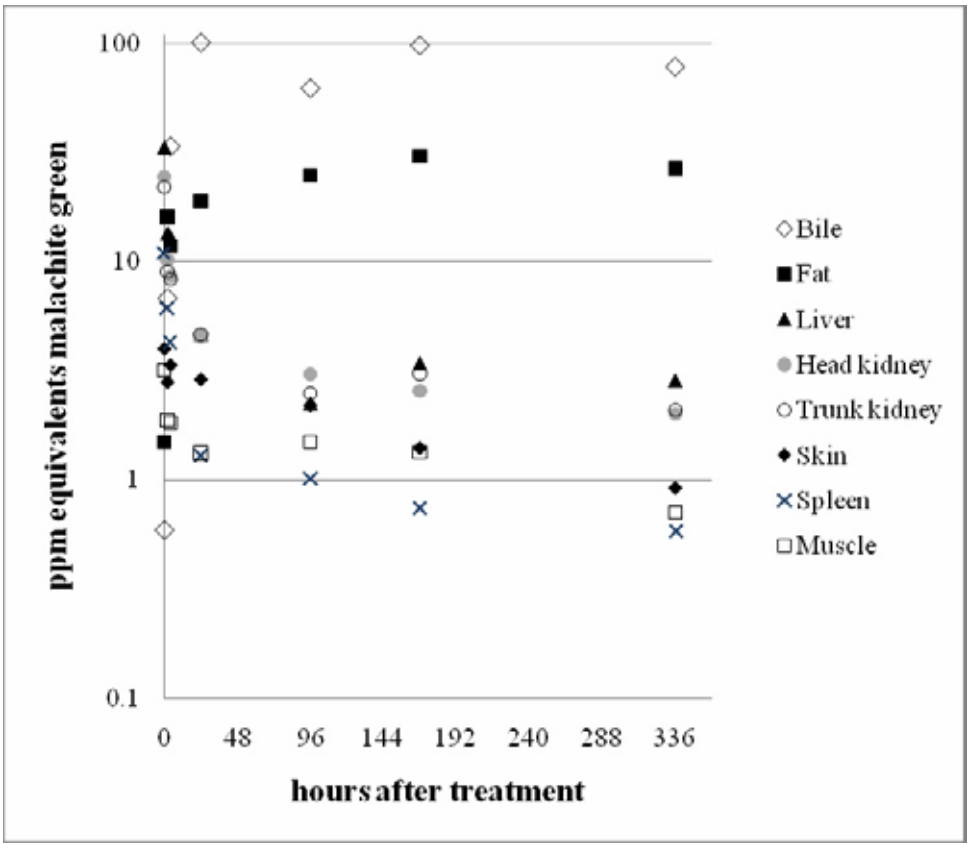
Malachite green and its metabolites were widely distributed and concentrated in the tissues. Concentrations of radioactive residues were highest in the excretory tissues and fat and lowest in the muscle and plasma. Concentrations of total residues exceeded the initial concentration of the water bath in all tissues, with the following few exceptions: bile (0 hours), spleen (168 and 336 hours), and muscle (336 hours). The authors' results, originally in tabular form, are summarized in figure 2 below. The variability of the results expressed as relative standard deviation of the mean of 5 data points ranged from 0.07 to 0.76. Variability was lowest in muscle tissue and highest in skin and bile. The variability increased over time. The high values of many standard deviations suggest that the residue concentrations might not be normally distributed and that the averages calculated by the authors are not the ideal parameters to show a central tendency. Concentrations of residues in skin were always higher than in muscle. The highest ratio was 2.2, observed 24 hours after treatment.

The concentrations of malachite green and of leucomalachite green in plasma were 3.29 and 1.94 µg/ml, respectively, immediately after dosing. One day after dosing, malachite green levels were at the LOQ while leucomalachite green levels were 0.11 µg/ml at day 14 after treatment.

In muscle, malachite green and leucomalachite green concentrations were 1.18 and 1.45 mg/kg, respectively, at the end of the exposure period and 0.012 and 0.52 mg/kg 14 days after treatment. Results obtained at other time points are not numerically given. The elimination of malachite green in muscle appeared biphasic with a terminal half-life of about 67 h. Concentrations of leucomalachite green were quantifiable for up to 42 days (0.02 mg/kg). Unidentified metabolites eluting before leucomalachite green during HPLC were found. The sum of the concentrations of these three metabolites reached a maximum of 31.3% of the total residue at 24 hours after treatment. No detailed pharmacokinetic information is provided for the water-borne exposure; however the authors state that the half lives for malachite green and for leucomalachite green were 2.8 and 10 days, respectively.

The effect of pH of the exposure solution was studied at pH values of 6, 7, and 8. When catfish were exposed to solutions of 0.8 mg/l of malachite green for one hour, uptakes increased significantly with increasing pH, determined by the concentrations of malachite green and leucomalachite green in plasma and muscle immediately after exposure. This may be due to the change in equilibrium concentrations of the cation and the carbinol and in the rates of conversion of the cation to the carbinol. The well designed and conducted study cannot be used for the derivation of MRLs because statistical evaluations are not possible in the absence of individual animal data.

Figure 2: Total residues of malachite green in tissues of channel catfish.



Pharmacokinetic studies in juvenile eels

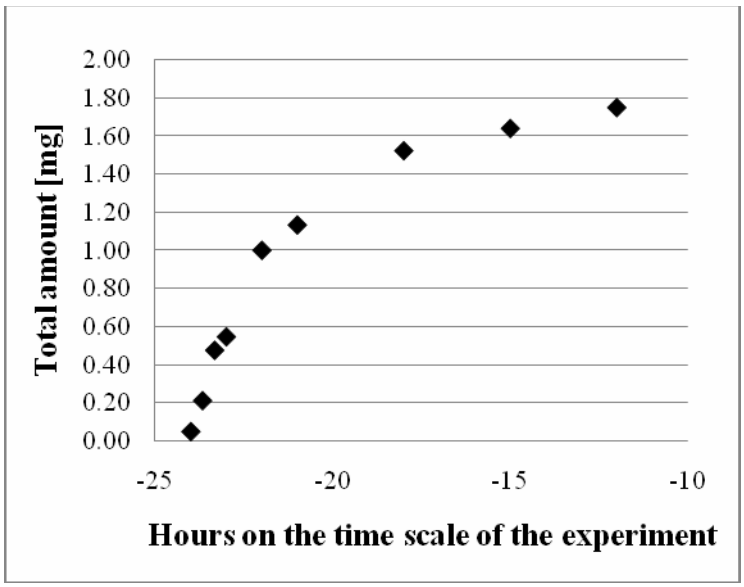
Bergwerf, et al. (2004) exposed 450 juvenile eels (*Anguilla anguilla*) of an average weight of 4.1 g (range 1.5 to 7.4 g) for 24 hours to malachite green in a water bath with temperature varying between 23.0 and 26.5°C and at pH values between 7.0 and 7.8 in 40 L water. A malachite green concentration of 0.1 mg/l was intended. Fish were then cultured in malachite green free water at 21.6 to 27.5°C. During this period pH varied between 6.0 and 7.8. Before, and at various time points during, and up to 100 days after treatment, ten fish and water were sampled (50 ml per time point). The fish did not grow during the study.

The whole fish were cut in fine pieces and 2g of cut tissues were blended in buffer and extracted with buffer/acetonitrile mixtures. Partitioning with dichloromethane was followed by solid phase extraction. Brilliant green was added as internal standard before HPLC analysis. Water samples were mixed with buffer, acetonitrile and internal standard prior to analysis. Two reversed phase columns (phenyl-hexyl and C_8) were used in series and were eluted with a mixture of 60% (vol/vol) acetonitrile and 40% (vol/vol) of 0.05M ammonium acetate buffer, pH 4.5, at 0.6 ml/min. The eluate was monitored at 620 nm after post column oxidation. Recoveries (n=36) were 61 ± 6% for malachite green and 88 ± 10% for leucomalachite green. Results were recovery-corrected.

Analysis of water revealed that the starting bath concentrations were only 0.032 mg/L instead of 0.1 mg/l. This concentration further decreased exponentially during the experiment and fell below the limit of detection at 12 hours. A definitive reason for the low initial concentration could not be found. The further decrease can apparently be explained by the uptake of malachite green by the fish. One can roughly estimate the total amount of malachite green taken up by the fish from the sum of the concentrations of malachite green and leucomalachite green found at a given time point multiplied with number and average weight of fish present in the bath at this time point plus the accumulated amounts removed from the bath by sampling 10 fish at every earlier time point. The figure 3 shows

the results of such a crude calculation. The total amount of drug initially present in the bath was estimated as approximately 1.28 mg using the information given by the authors. The small discrepancies are probably due to the crude estimates used in these calculations.

Figure 3: Estimated time course of the exhaustive uptake of malachite green in the exposure experiment.



The density of fish in the small bath which absorbed the drug and the long exposure time probably caused a total uptake of the malachite green present. Even the higher originally intended concentration would have been too low for such an experiment. The figures 4a and 4b summarize the pharmacokinetic results of the experiment. The symbols represent mean values. The bars show the range. If a lower bar extends down to 0.2 µg/kg, the result of the analysis was <LOD. The LOD was given as 0.2 µg/kg.

Figure 4a: Concentration changes of malachite green and leucomalachite green during and after bath exposure to 0.032 mg/L of malachite green.

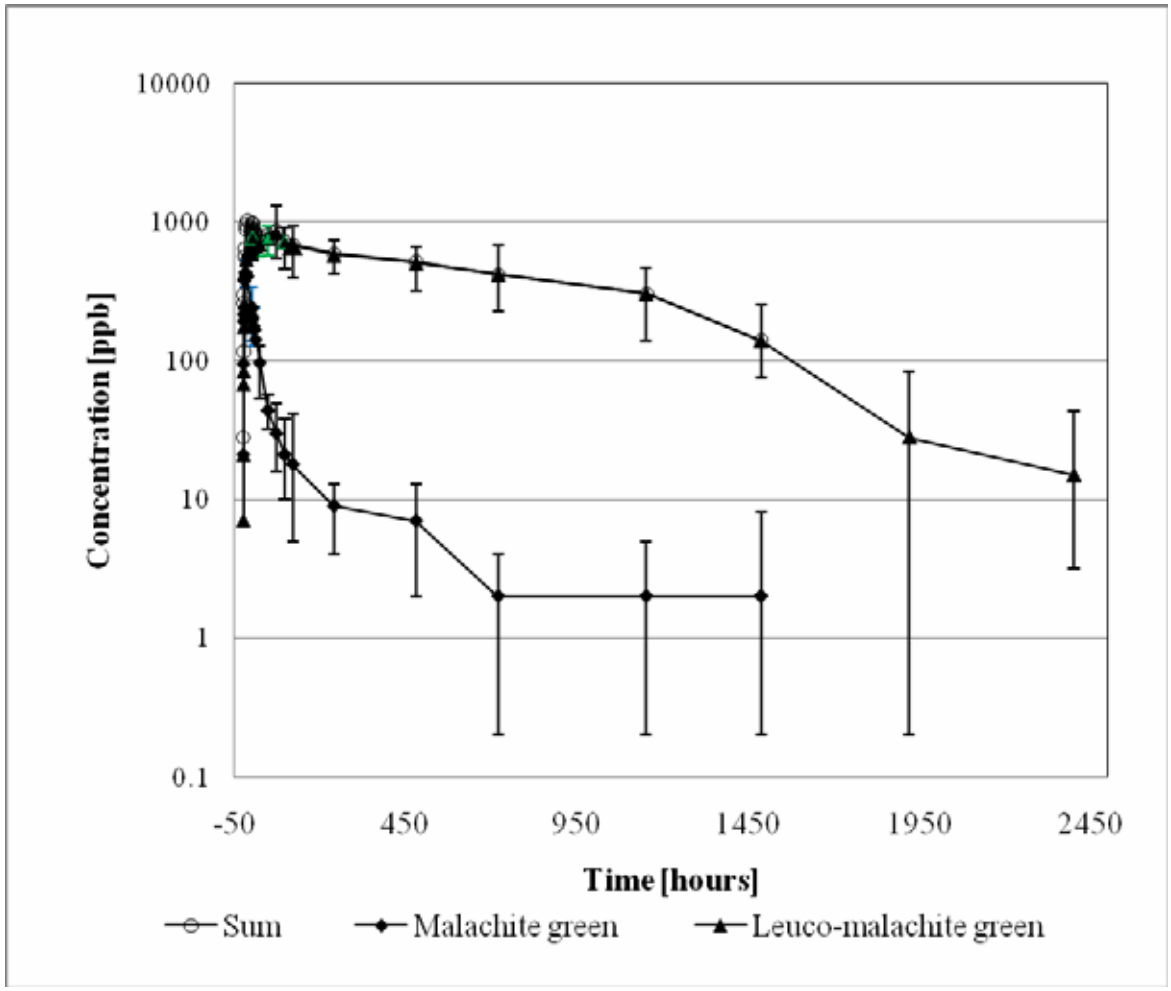
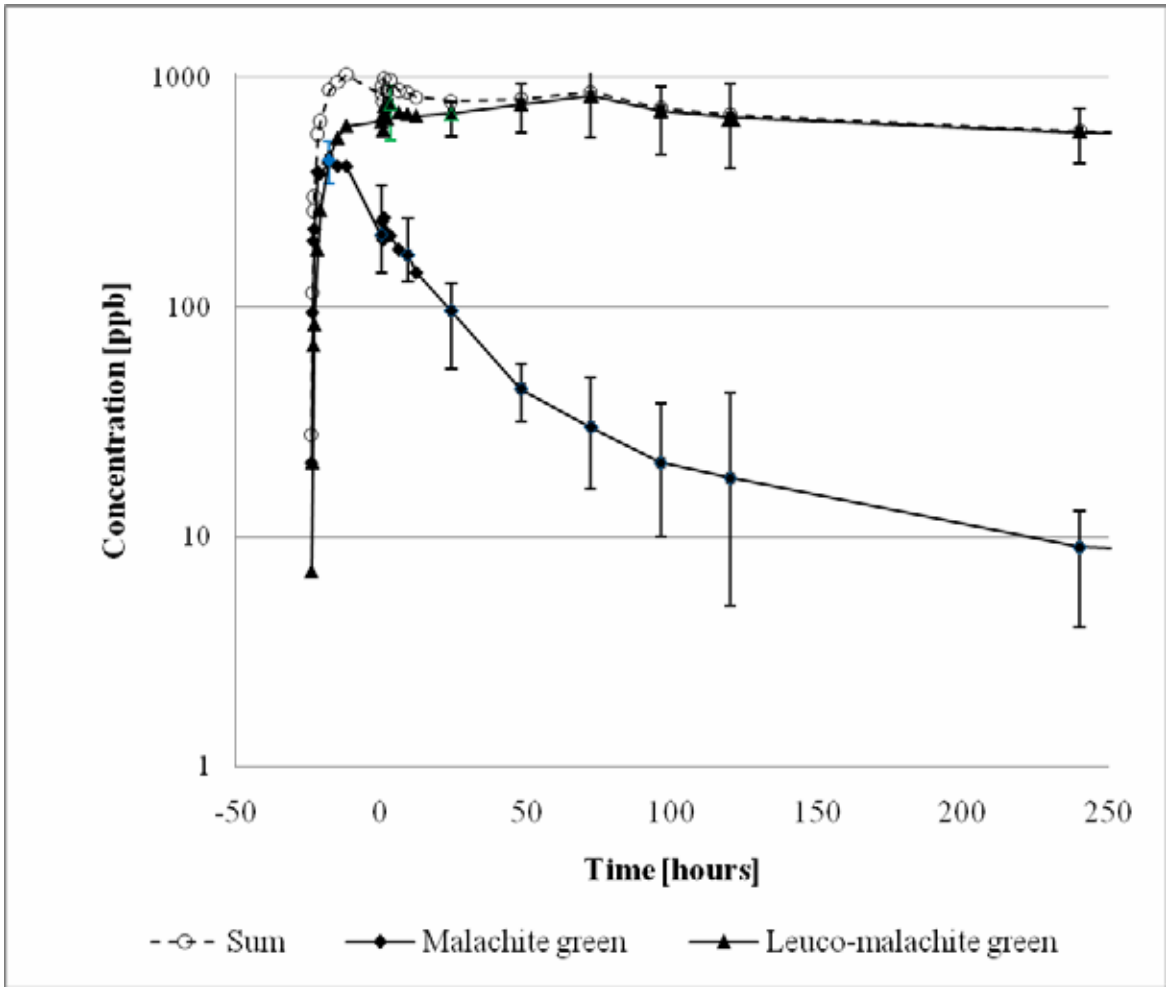


Figure 4a shows the averages of the measurements obtained with 9-10 individual fish at every time point and the range of the individual values. It is not clear how the averages have been calculated at time points where "non-detects" occurred. The latest time point at which the results were not influenced by "non-detects" was 480 hours for malachite green and 1487 hours for leucomalachite green. Malachite green was quantified in the one or other fish up until day 62 following exposure and leucomalachite green was found in the one or other fish over the whole 100 days observation period following exposure. This finding is important in view of the very limited dose administered.

Figure 4b: The exposure and early depletion part of figure 4a.



For graphical reasons figure 4b shows bars indicating ranges of individual observations only for selected time points well spaced on the time scale. The figure shows that almost immediately with the uptake of malachite green at the beginning of the exposure period also the concentration of leucomalachite green starts increasing. Due to the circumstances described above, the data of this study are neither useful for pharmacokinetic evaluations nor any further interpretations.

METABOLISM

Metabolism in Micro-organisms

Fungi

Some ligninolytic fungi have been found capable of decolorizing synthetic dyes. This is due to their production of enzymes such as, laccase and Mn-peroxidase that enable these microorganisms to oxidize a broad range of substrates. Studies have focused on the possible use of some model wood-rotting white-rot species (*Phanerochaete chrysosporium*, *Trametes versicolor*, *Pleurotus ostreatus* and others) for decolorization of synthetic dyes. Ligninolytic cultures of the white rot fungus *Phanerochaete chrysosporium* were shown to metabolize crystal violet and malachite green to N-demethylated metabolites catalyzed by lignin peroxidase. Extracellular fluid obtained from ligninolytic cultures of this fungus, retained the activity provided that an H_2O_2-generating system was supplied. Non-ligninolytic (nitrogen-sufficient) cultures also degrade crystal violet by another mechanism without producing N-demethylated metabolites (Bumpus and Brock, 1988). Eichlerova, et al. (2006a) investigated the dye decolorization capacity of two white rot fungi, *Dichomitus squalens*

and *Ischnoderma resinosum* (Eichlerova, et al., 2006b). *D. squalens* showed high decolorizing capacity. *I. resinosum* decolorized malachite green to a lower extent up to a concentration of 0.1 g/l.

A total of 26 white rot fungi from Argentina were tested for their ability to produce lignin-modifying enzymes and decolorize industrial dyes (Levin, et al., 2004). Ten of the strains decolourised all tested dyes including malachite green. The mycelia were grown on solid malt extract/glucose media containing the dye. All ten strains produced laccase, lignin peroxidase and manganese peroxidase on solid medium. White-rot fungi normally require a lignocellulose substrate. Their use in polluted water streams or soils may be problematic since species of these typical wood colonizers do not exhibit satisfactory growth and competitiveness under such conditions.

Litter-decomposing fungi differ from wood-rotting species with respect to their growth substrate, forest litter and soil. They are characterized by higher C:N ratio and microbial activity. Laccase is the most common ligninolytic enzyme among these organisms and Mn-peroxidase is produced only by some species (Baldrian and Snijdr, 2006).

Cha, et al. (2001) performed biotransformation experiments of malachite green with cultures of *Cunninghamella elegans*, a filamentous fungus which had previously been shown to enzymatically catalyse N-demethylation and N-oxidation reactions of a number of chemicals. Metabolites were analysed using HPLC-diode array and HPLC-MS methods. Malachite green was reduced to leucomalachite green and also converted to N-demethylated and N-oxidized metabolites, including primary and secondary arylamines. The mono-, di- and tri-desmethyl derivatives of malachite green and the mono-, di-, tri-, and tetra-desmethyl derivatives of leucomalachite green were found in the supernatant following removal of the mycelium. Malachite green N-oxide was only detected in the mycelia. Identical patterns of metabolites were observed with malachite green and with leucomalachite green as initial substrate. After prolonged incubation only reduced metabolites were found suggesting that parent malachite green and N-demethylated metabolites were reduced by the fungus. Microsomal fractions did not produce reduced metabolites in the absence of NADPH. The cytochrome P450 inhibitor metapyrone completely inhibited the biotransformation reactions.

Intestinal bacteria

Henderson, et al. (1997) studied the metabolism of malachite green by intestinal microflora from human, rat, mouse, and monkey fecal samples and 14 pure cultures of anaerobic bacteria representative of those found in the human gastrointestinal tract. All complete microfloras were very efficient in reducing malachite green to leucomalachite green (human and rhesus monkey intestinal microfloras, C3H/HEN-MTV mouse intestinal microflora, and Fisher 344 rat intestinal microflora). Of the bacteria commonly found in the human intestinal tract, *Clostridium perfringens (ATCC 3624)*, *Escherichia coli (ATCC 25922)*, and *Peptostreptococcus anaerobius (ATCC 27337)* converted almost all of the dye to the leuco derivative. The conversion was monitored with HPLC with diode array detection and the structure was confirmed by mass spectrometry.

Baker's yeast (*Saccharomyces cerevisiae (MTCC 463)* was also shown to effectively decolorize malachite green, primarily through reductive pathways (Jadhav and Goindwar, 2006). A number of other bacteria have been positively tested for decolorizing capacity of malachite green. A complete review would go beyond the scope of this monograph.

Metabolism in Laboratory Animals

Rats and mice

In short term feeding studies, Culp, et al. (1999) have shown that MG is sequentially *N*-demethylated to secondary and primary aromatic amines in rats and mice both before and after reduction to LMG. Female and male B6C3F_1 mice and Fischer 344 rats were fed up to 1200 mg/kg malachite green or 1160 mg/kg leucomalachite green for 28 days. The malachite green used was ≥ 94% pure. Impurities

detected were leucomalachite green (1%) and demethlyated derivatives of malachite green (3.5%). Leucomalachite green was ≥ 98% pure. Impurities detected were malachite green and mono-desmethyl leucomalachite green. Livers were extracted using a modification of a published method (Roybal, et al., 1995). The extracts were analysed by HPLC connected to a post-column oxidation chamber and a photodiode array detector. Analyses using HPLC-APCI/MS also were performed. The desmethyl derivatives were synthesized to confirm structures in the samples subjected to APCI/MS.

In HPLC-APC/MS analysis of liver extracts from rats treated with leucomalachite green the primarily seen compounds were protonated leucomalachite green, protonated demethylated derivatives and the molecular ions of malachite green N-oxide and demethylated N-oxide. A small, but measurable, amount of malachite green was also found. At higher cone voltages additional collision-induced diagnostic fragments were found that were formed following losses of dimethylaniline-, methyl-, or phenyl- moieties. The appearance of these molecules was consistent with the fragmentation pathways previously published for leucomalachite green (Doerge, et al., 1998a) who observed similar sequential demethylation in a thyroid peroxidase-catalyzed reaction of leucomalachite green. A dose-related increase in leucomalachite green and metabolites was observed in both rat and mouse liver extracts.

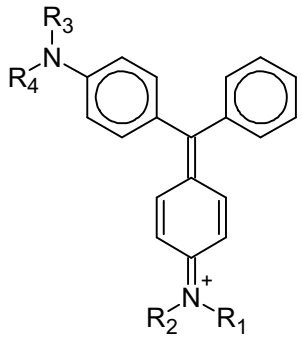
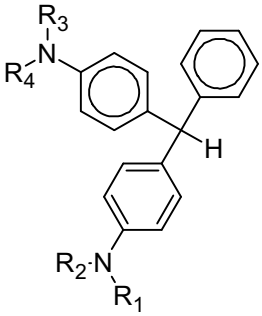
Similarly, HPLC-APC/MS analysis of liver extracts from rats treated with malachite green detected the molecular ions for malachite green, its mono-, di-, tri-, and tetra-desmethyl derivatives, and malachite green *N*-oxide. A small, but measurable, amount of leucomalachite green was also detected. Higher cone voltages produced fragments consistent with those previously reported by Doerge, et al., (1998b). These authors incubated leucomalachite green with tyrosin peroxidase, iodide, and tyrosine in the presence of an H_2O_2 generating system and obtained the mono-, di-, and tri—desmethyl derivatives of leucomalachite green as well as malachite green and malachite green-N-oxide. Concentrations of malachite green and metabolites increased with increasing dose.

The formation of both the symmetric and asymmetric di-desmethyl malachite green metabolite could be demonstrated with the symmetrical isomer eluting first. When liver extracts were analysed using HPLC/UV detection, leucomalachite green was the major product detected in rats fed leucomalachite green (accompanied by small amounts of mono- and di-desmethyl-leucomalachite green) and malachite green was the major product detected in the livers of rats and mice fed malachite green (accompanied by mono- and di-desmethyl malachite green and leucomalachite green – and in the case of rats mono- and di-desmethyl-leucomalachite green).

^{32}P-Postlabeling of liver DNA indicated the formation of a DNA adduct, or co-eluting adducts, that increased with increasing dose, in rats and mice fed leucomalachite green or malachite green. Cho, et al. (2003) mention that malachite green and the *N*-demethylated derivatives of malachite green and leucomalachite green are capable of forming DNA adducts *in vivo*, with the binding being consistently greater with the ionic MG derivatives.

Figure 5 below summarizes the structural elements of the N-desmethyl metabolites of malachite green and leucomalachite green.

Figure 5: Structures of malachite green and leucomalachite green.

Structures of malachite green, leucomalachite green, and demethylated derivatives								
	Malachite green				Leucomalachite green			
	R_1	R_2	R_3	R_4	R_1	R_2	R_3	R_4
Parent molecule	CH_3	CH_3	CH_3	CH_3	CH_3	CH_3	CH_3	CH_3
Desmethyl-	CH_3	CH_3	CH_3	H	CH_3	CH_3	CH_3	H
Di-desmethyl- (symmmetric)	CH_3	H	CH_3	H	CH_3	H	CH_3	H
Tri-desmethyl-	CH_3	H	H	H	CH_3	H	H	H
Tetra-desmethyl-	H	H	H	H	H	H	H	H

Metabolism in Food Producing Animals

Fish

No systematic metabolism study has been performed in fish. However, many of the degradation products formed either under physical-chemical conditions or in *in vitro* and *in vivo* studies in bacteria, fungi and laboratory animals have also been found in fish. The potential presence of such molecules in monitoring samples is largely ignored by analysts and only a few methods use protective agents in order to prevent the breakdown of incurred residues during extraction and cleanup.

TISSUE RESIDUE DEPLETION STUDIES

Fish

Poe and Wilson (1983) reported in a short note to the journal "Progressive Fish Culturist" that they had observed a green color developing after some storage time on the surface of the tissue of skinned frozen fish. The color could be extracted using a procedure for lipid extraction and was identified as malachite green by infrared and visible spectra. They conducted a small series of experiments with channel catfish of body weights ranging from 0.3 to 0.7 kg in which they varied exposure conditions (exposure levels and frequencies). Samples of visceral fat and the carcass were frozen. The green color appeared typically in visceral fat more rapidly than in muscle. When Alderman published his frequently cited article "Malachite green: a review" in 1985, uptake and residues had not yet become a significant issue in the scientific literature.

Eggs and fry

Allen and Hunn (1986) reported that malachite green accumulates in the eggs of gravid female salmon after treatment and is detectable in eggs and newly hatched fry.

Meinertz, et al. (2005) determined residues of [^{14}C]-malachite green in eggs and fry of rainbow trout after treatment of the eggs. The treatment method was flush treatment (a concentrated solution of malachite green was added to an incubation unit and flushed through with fresh water). At the beginning of the treatment the eggs were approximately 2 days old. Hatching began on day 25 and

was completed on day 31. Six groups of 250 eggs each were treated in 500-ml glass test aquaria. Before treatment water flow to the aquaria was stopped and the volume was drawn down to 265 ml. Eggs in all aquaria were exposed simultaneously using a distribution manifold. The expected nominal concentration was 1.0 mg/l. Water flow was re-established immediately after treatment. Treatment was performed on days 0, 3, 6, 9, 12, 15, 18, 21, 24, and 31. Water samples were taken at every treatment from one randomly selected aquarium immediately after addition of the treatment solution and 2.5, 5, 10, 15, 20, 25, 30, and 60 minutes after water flow was re-established.

Ten eggs were sampled from each test aquarium immediately before each treatment through day 24. Five were prepared for combustion analysis (92.8 to 95.8 combustion efficiency) and the remaining five were used for analysis of malachite green residues by HPLC with post-column oxidation and visible light detection. On day 31, 10 fry were sampled from each aquarium immediately before the final treatment and 0, 6, 12, and 24 hours, and 2, 4, 7, 12, 17, 22, and 28 days after treatment. Mean peak concentrations of chromatic malachite green in water were 0.37 mg/l and were slightly higher than the radioactive concentration equivalents. The intended nominal concentration was not reached and individual measurements were extremely variable at all time points.

Untreated eggs contained measurable concentrations of unlabelled malachite green. Pre-treatment radioactive concentration equivalents in eggs and fry increased from day 0 to day 31 to a concentration of 271 ± 42 (n=6) – without exhibiting saturation effects or reaching a steady state - and declined to 55 ± 11 on day 28 after the final treatment. The efficiency of the analytical method to extract radioactivity was 49 to 119 % (average 76%). Leucomalachite green was the predominant residue. OD and radioactivity traces of HPLC separations showed one more polar unknown compound in addition to the known compounds. If measurements in fry were corrected for growth, the elimination half life was 9.7 days.

Kinetic depletion studies in fish

In addition to the studies described in the section of pharmacokinetics some other studies have been performed. Bauer, et al. (1988) published an article “Uptake and excretion of malachite green in rainbow trout”. They described an HPLC method for the determination of malachite green and leucomalachite green. Recoveries were approximately 75% and all results were recovery corrected. For the experiment they used 156 rainbow trout with an approximate body weight range of 200-300 g. The total weight of all fish was 41.2 kg. The temperature was 9.7 ± 0.1°C. Treatment was performed in a tank with 1000 l water to which 200 mg malachite green was added. The proportions of fish to water corresponded to intensive aquaculture conditions for trout. Duration of treatment was 24 hours. Samples for water analysis were taken every hour. Ten fish were sampled immediately after the end of treatment and groups of six were sacrificed at all other sampling times. The last samples were taken on day 143 after treatment. Homogenized tissue samples were frozen and stored at -30°C prior to analysis. Two parallel smaller experiments were carried out in smaller tanks and with smaller fish densities and a treatment concentration of 0.1 mg/l. for methodological studies.

The initial concentration in water of malachite green was 205 mg/m^3 and decreased to 5 mg/m^3 in 24 hours following an exponential term. Thus 97.6% of the malachite green disappeared and was probably taken up by the fish because in one of the smaller experiments 80% of the malachite green that had disappeared from the water bath was found in the fish. About 33% of the malachite green which was taken up was found in muscle. At the end of the treatment period the total concentration of malachite green plus leucomalachite green was 910 ± 243 µg/kg (n=10). The concentration of the parent drug was 86.3 ± 54.4 µg/kg (n=6). On subsequent days the concentrations of the parent drug rapidly decreased and the between fish variability increased.

A graph provided in the original paper shows that the decrease in the concentration of malachite green did not follow a mono-exponential term. However, the group of data points describing the depletion of the leucomalachite green comes closer to a log-linear curve. The concentrations measured in the fatty tissue were very high. Therefore the authors determined the fat content of the muscle samples.

For the muscle samples taken during the first 87 days classified according to fat content they found a very high correlation between fat content and concentration of leucomalachite green in muscle and a decrease of the rate of depletion of leucomalachite green in the groups of fish with the highest fat contents. For fish with the highest fat content the elimination half life of leucomalachite green was 43.3 days. The almost complete uptake of the malachite green shows that the compartment fish was still far from any saturation in this experiment.

Allen (1990) applied colorimetric analysis to samples of muscle, eggs and fry of malachite green treated Atlantic Salmon (*Salmo salar*) and Chinook Salmon (*Oncorhynchus tshawytscha*). Fish had been treated 10 to 47 times with a solution containing 1 mg/kg of malachite green oxalate for one hour. Samples were obtained 1 to 18 days after the last treatment. Residues were extracted with a mixture of 85% ethyl alcohol, 10% formalin and 5% acetic acid. Following extraction in the dark, centrifugation and filtering absorbance at 615 nm was measured in the extracts. The method was not validated. The author states that concentrations of residues in muscle of Atlantic salmon showed no relation with the number of treatments and the concentrations in both species depended only on the elapsed time after the last treatment. Since the methodology is inadequate for the determination of malachite green the numerical results published by the author are most likely of little value.

A paper in Thai language (Amornchai Somletchaoen) for which only an English summary is available reports on the persistence of malachite green in tilapia. Sixty juvenile tilapias of an average body weight of 24.1 ± 6.8 g were exposed to malachite green at two therapeutic doses, 0.1 for 24 h and 0.2 for 1 h at a water temperature varying between 23.5 and 26.0°C. The fish were then transferred into clean water and 3 fish were collected at 0, 6, 12, 24, 72, 120, 168 and 360 h post exposure for the determination of malachite green and leucomalachite green residues in muscle tissues. A LC-MS-MS method was used with LOD of 2µg/kg. Following treatment with the high therapeutic dose, highest average concentrations of malachite green and leucomalachite green were 35.6 ± 5.8 and 32.2 ± 17.5 µg/kg, respectively. Malachite green depleted to 0.4 ± 0.15 µg/kg within 24 hours while leucomalachite green was 1.5 ± 0.7 µg/kg after 120 hours. After treatment at the lower dose the highest average concentration of malachite green and leucomalachite green 4.6 ± 1.8 and 30.6 ± 2.6 µg/kg, respectively. The concentration of malachite green was 2.0 ± 0.35 µg/kg at 72 h and not detectable 168 h after treatment. Concentrations of leucomalachite green remained stable between 12 to 72 h after treatment. At 360 h after exposure, the average concentration was 3.5 ± 2.3 µg/kg.

A study investigating the metabolic profiles and residues of malachite green in trout tissues was carried out for the United States Food and Drug Administration (Law, 1994). The study was conducted in trout kept in tanks under the following conditions: water temperature (10±2°C), pH (6.0-7.0), hardness (5-10 mg/l), and dissolved oxygen (9±2 mg/l). ^{14}C-Labeled malachite green of a radiochemical purity of 98% was used for the treatment. All experiments and analytical work was carried out under decreased intensity room light. Concentrations in the exposure tanks were maintained by a metering apparatus containing a ^{14}C-MG stock solution at 800 mg/l and delivering 10 ml/min of this solution; the concentration of the treatment solution was 2 mg/kg.

Seventy-two randomly selected trout, each weighing about 350g, were divided into 3 groups of 24 fish and put into three 200-l continuous flow exposure tanks containing 2.0 mg/kg ^{14}C-labeled MG (actual concentrations 1.8 ± 0.2 mg/kg, 1.9 ± 0.3 mg/kg and 1.9 ± 0.2 mg/kg, respectively). A water sample (5 ml) was withdrawn from the exposure tanks every 15 min during the ^{14}C-labeled MG exposure period. After a 1-h exposure, the fish were removed to a depuration tank containing flowing, uncontaminated water. At specific time intervals during ^{14}C-labeled MG exposure and depuration two or three trout were removed randomly from each group of fish and sacrificed. The annexes to the study report provided information on concentration of total radioactive residue in tissue homogenates and ratio of malachite green to leucomalachite green concentrations in an organic extract. From these data the concentrations of malachite green and leucomalachite green in the tissues were calculated. The highest concentrations of residues were found in liver and kidney. Significant concentrations of residues were also found in skin.

The data of the study by Law (1994) representing the time period between the end of the treatment and 505 hours post treatment were subjected to statistical analysis using one exponential term on the basis of the natural logarithms of the residue contents for curve fitting. The following parameters given in Table 3 were obtained by linear regression:

Table 3: Parameters of the linear regression analysis of kinetic residue depletion data in trout muscle.

Parameter	MG	LMG
Intercept	- 0.99747	- 0.01994
Slope	- 0.02461	- 0.00352
Coefficient of correlation	- 0.60012	- 0.73361
Residual variance	0.58312	0.52876

The kinetic data representing the concentrations of malachite green and leucomalachite green between the end of the treatment and 505 hours were subjected to statistical analysis using one exponential term on the basis of the natural logarithms of the residue contents. Depletion half lives were 28 hours for malachite green and 197 hours for leucomalachite green. The kinetic parameters including the variance of the data were used to perform estimates of dietary exposure to malachite green (see below). The results obtained for muscle are summarised in Figures 6 and 7. The data are the same in the two figures. In Figure 6 the time axis is given on a logarithmic scale in order to enable better discrimination of the treatment phase and the phase after treatment.

Figure 6: Kinetics of malachite green and leucomalachite green in trout muscle.

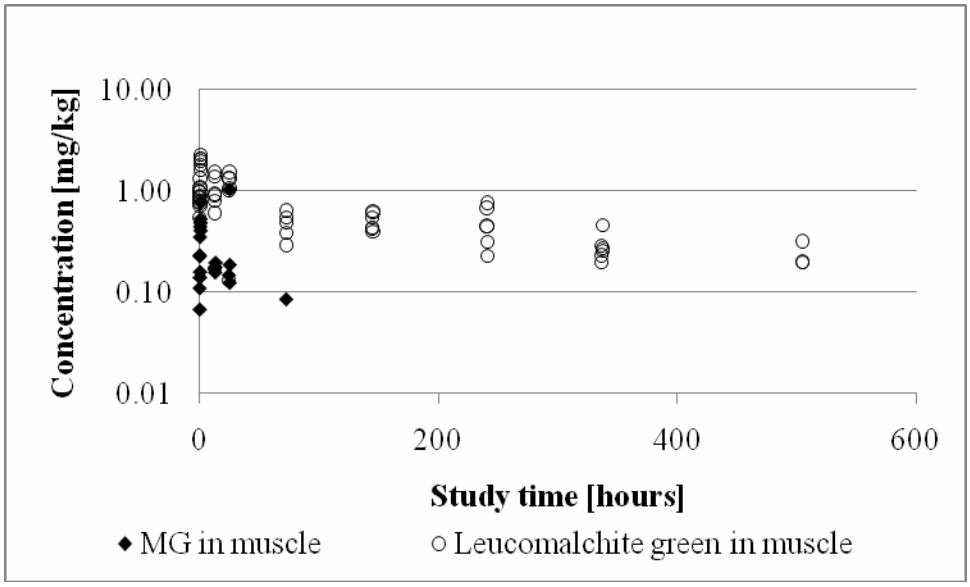
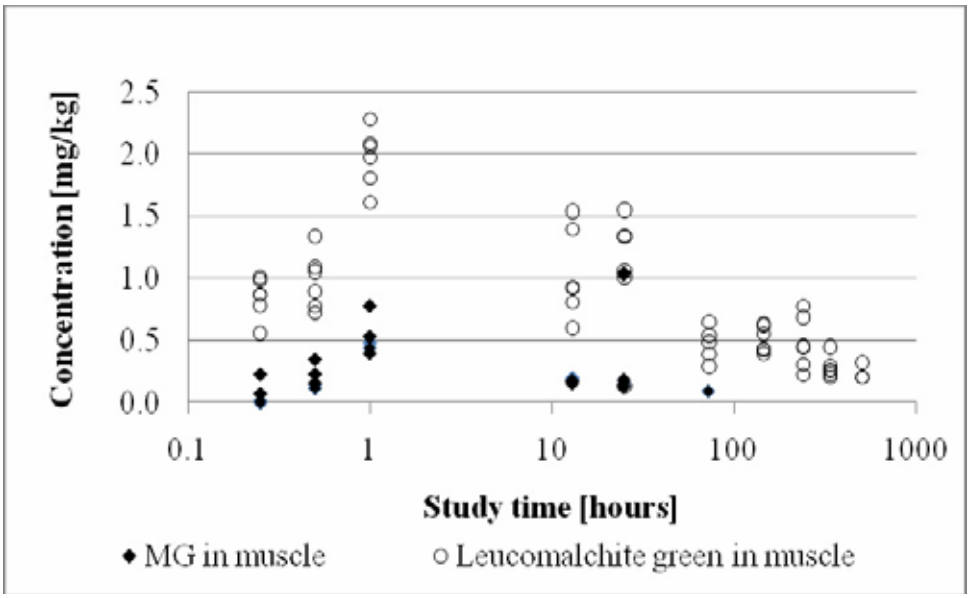


Figure 7: Kinetics in trout muscle – logarithmic time scale.



The following table of results of trials is compiled from the above mentioned publication of Sudova, et al. (2007) that cites the data after Mitrowska and Posyniak (2005).

Table 4: A selection of treatment conditions cited after Mitrowska and Posyniak (2005).

Fish species	Average body weight	Duration of bath [hours]	Water temperature [°C]	pH of bath	Time after treatment [days]	Concentration in muscle tissue [µg/kg]	
						Malachite green	Leucomalachite green
Eel	4.1	24	25	6.9	62	2	139
					80	< LOD	28
					100		15
	0.3				330		< LOD
Channel catfish	600	1	21	7.1	14	12	518
					62	< LOD	19
	580		22	7.2	14	6	310
Rainbow trout	1350		21	7.0	1	73	289
			12	7.8	5	15	230
	0.1	72	9.7		40	1	20
					140	< LOD	
		144	13		300		2

Unsystematic small trials conducted in the context of method development studies

Some information on residue behavior was generated in a less systematic manner in the context of the development of analytical methods. The below section summarized a selection of these studies.

Allen, et al. (1994) carried out recovery experiments in order to assess the performance of a method and treated 6 adult rainbow trout (range of body weights was 1200-1500 g) in well water of pH 7.8 with 1 mg/l of ^{14}C malachite green for 1h . Residues were determined by combustion analysis in fillets (with skin left on) immediately after exposure and after 5 days withdrawal period. Both fortified homogenates and homogenates from treated fish were extracted and analysed after cleanup using HPLC, collection of radioactive fractions and liquid scintillation counting. The results are summarised in Table 5.

Table 5: Results of the study of Allen, et al. (1994).

Material	Days after treatment	Original concentration [mg/kg]		Found in extract		Composition of extract [%]		
		Total residue after combustion	Fortification level with MG	[mg/kg]	%[1]	MG	LMG	Unknown
Fish 1, muscle	0	1.3		0.8	62	29	45	25
Fish 2, muscle				1.1	85	26	49	24
Fish 3, muscle				1.0	77	34	45	21
Fish 4, muscle	5	0.5		0.5	100	3.0	46	51
Fish 5, muscle				0.3	60	3.0	33	64
Fish 6, muscle				0.3	60	1.8	40	59
Egg homogenate			1		85	84	7	9
			0.5		98	81	10	9
Fry homogenate			0.65		68	76	11	13
Muscle homogenate			1		66	11	89	

[1.] The values for the six incurred tissues are calculated from the data; they are not given in the original paper.

The following results obtained with trout muscle are interesting:

- Recoveries from incurred muscle tissues were lower than from fortified homogenates;
- A significant fraction of the radioactive residue was of unknown structure. This fraction increased from approximately 23% immediately after treatment to approximately 58% on day five after treatment.
- Malachite green added to homogenates was largely reduced to leucomalachite green.
- The original solution of malachite green remained unchanged if processed by the same procedure in the absence of tissue homogenate.

Andersen, et al. (2005) developed an analytical method based on liquid chromatography in which leucomalachite green is oxidised prior to cleanup with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ). Most of the DDQ is consumed by the sample extract. A high excess is needed; however, excess oxidation and formation of breakdown products has to be avoided. The study included the determination of residues in salmon that had been treated with malachite green. Fish (weight not given) were treated in a water bath (volume not given) with 0.01 mg/l malachite green for one hour (temperature not given). The fish were returned to clean water and sampled 2 and 4 hours after the end of exposure. The concentrations (sum of malachite green and leucomalachite green) were found in Table 6. The authors discuss the lower recoveries of spiked malachite green compared to leucomalachite green as a possible explanation of the higher concentrations found at the later time point. Mean recoveries for malachite green were approximately 71% over a concentration range of 1-10 µg/kg. For the same concentrations of leucomalachite green recoveries averaged 95.4%.

Table 6: Results of the study of Andersen, et al. (2005).

Hours after treatment	Concentration [µg/kg]			
2	29.8	35.2	36.3	35.3
4	48.0	47.6	36.9	52.5

The same group published a paper in which a similar method was used, but results were confirmed with LC-MS (Andersen, et al., 2006). The method was validated for catfish, trout, tilapia, basa, salmon, and shrimp. Incurred tissues were also analysed. For this purpose catfish, tilapia, trout, and salmon were exposed to 0.01 mg/l malachite green in water for 1 h. After transfer to clean water samples were analysed at a different time point for each species 16, 16.25, 16.5, and 24 h, respectively, after the end of the treatment. The results are summarised below:

Table 7: Results of the study of Andersen, et al. (2006).

Fish species	Hours after treatment	LC-VIS		LC-MS	
		mean	sd	mean	sd
		[µg/kg]			
catfish	16	32.2	2.2	31.3	2.7
tilapia	16.25	1.9	0.13	2.1	0.3
trout	16.5	27.1	1.4	28.6	1.1
salmon	24	26.4	0.84	27.4	2

Bergwerf and Scherpenisse (2003) published a method for the determination of malachite green in aquatic animals which is based on HPLC or LC-ESI-MS-MS (for confirmation). The mobile phase was pumped through a pre-column oxidation reactor and effluent was oxidised in a post-column reactor. The authors made the observation that the time interval between spiking of a homogenate and further processing influenced the recovery of malachite green significantly, but had little influence on recoveries of leucomalachite green.

Table 8: Results of the study of Bergwerf and Scherpenisse (2003)

Time [min]	Recovery [%]	
	Malachite green	Leucomalachite green
1	81	100
15	63	96
30	60	93
60	54	93
120	45	95

Malachite green was apparently not reduced, but possibly degraded, since the chromatograms showed satellite peaks next to malachite green. The method was optimised for leucomalachite green. Forty-eight samples of trout, eel and salmon were collected at retail level and on fish markets. Approximately 50% were tested positive for leucomalachite green.

Doerge, et al. (1998b) described LC methods for the simultaneous quantification of malachite green and leucomalachite green using isotope dilution mass spectrometry. In addition they characterised metabolites derived from malachite green and leucomalachite green found in catfish and trout. Mature catfish of approximately 0.5 kg bw were exposed for one hour in a 40 l tank to 1 mg/kg malachite green at 25°C and pH 7.2. The fish were briefly rinsed and transferred to fresh clean water. Fish were killed 24 hours after dosing and fillets (skin removed) were blended and stored at -60°C prior to

analysis. Leucomalachite and malachite concentrations in muscle of treated catfish were 1030 and 590 µg/kg, respectively. Trout were purchased in 1994-1995 from retail outlets in the UK. Blended tissues were spiked with d5-leucomalachite green and $^{13}C_6$-malachite green. Recoveries of the internal standards were about 34 – 70% (n=12) for malachite green and 64-86 % (n=12) for leucomalachite green. The concentrations of incurred residues ranged from 0.4-3.4 µg/kg for malachite green and 9-96 µg/kg for leucomalachite green. Leucomalachite green was present at much higher concentrations (range 12- to 38-fold).

Halme, et al. (2007) proposed an LC-ESI-MS/MS method for confirmation of residues of malachite green and leucomalachite green in trout. D5-leucomalachite green was used as internal standard. They analysed 34 fish monitoring samples of which eight contained malachite green residues. Only the range of the results is given (0.35-1.34 µg/kg of leucomalachite green).

Roybal, et al. (1995) developed a method for the determination of malachite green and leucomalachite green by SPE, HPLC, post-column oxidation and detection at 618 nm. In this context they analysed catfish exposed to 1 mg/kg malachite green oxalate for 1h at 21°C and pH 7.0. The treated and rinsed fish were place into separate aquaria equipped with activated carbon filters. Fish were sacrificed and analysed at 0, 2, 4, 8, and 24 hours after placement in individual aquaria.

Table 9: Results of the study of Roybal, et al. (1995).

Hours after treatment	Concentration [µg/kg]				Replicates
	Malachite green		Leucomalachite green		
	mean	s.d.	mean	s.d.	
0	486	23.4	632	23.6	4
2	190	18.8	703	30.8	4
4	187	23.7	748	30.0	4
8	111	12.8	450	30.7	4
24	73.4	7.5	289	19.8	4

Scherpenisse and Bergwerff (2005) published a method for the determination of residues of malachite green in finfish by LC-MS/MS. Recoveries for malachite green were very low in most fish matrices. Recoveries for leucomalachite green were from 86 to 105%. They used the method to analyse nineteen samples including pangasius, salmon, shrimps and trout bought in local shops. Residues were found in three of the samples (trout 24 and 0.15 µg/kg, pangasius 7 µg/kg).

Turnipseed, et al. (2005) proposed an analytical method in which leucomalachite green is oxidised to malachite green before the SPE extraction step of cleanup and final LC-MS determination. They used the method to analyse two samples of treated salmon (10 µg/l for 1 hour). They found 34.6 µg/kg in a fish 2h after treatment and 44.3 µg/kg in a fish 4 h after treatment.

ESTIMATION OF DAILY INTAKE

In the open literature, well conducted residue studies suitable to predict the concentration–time course of residues of MG in fish are available for only two species, the rainbow trout and the channel catfish. Only for trout were sufficient individual animal data available to perform a statistical evaluation.

Useful information on frequency of occurrence and levels of residues can primarily be obtained from monitoring activities or from well supervised trials conducted under field conditions. The following discussion analyses the selected data for estimation of exposure.

In the UK, approximately 400 trout samples were analyzed in three surveys between July 1993 and March 1995. Sixty-seven samples contained malachite green at concentrations of 2-50 µg/kg. The analytical method did not pick up leucomalachite green. In a survey of retail trout in 1996, malachite

green was detected in 15 of 208 samples. In 1997 there was only one trout sample out of 137 that contained malachite green. A change in methodology was introduced in 1997 and subsequently malachite green and leucomalachite green were measured. When thirty-one randomly taken samples of the 1997 survey of which 29 were negative were re-analyzed with the new method, seven became positive. The new method was applied to the 27 samples taken in 1998. One contained both malachite green and leucomalachite green. In five samples only leucomalachite green was found (COT, 1999). Thus, the introduction of new methodology increased the number of positives. The individual results of the non-compliant samples are given in the cited COT document.

The Veterinary Residues Committee established in 2001 in the UK published the results of all statutory and non-statutory surveillance schemes. For non-compliant samples the individual numerical values found are also given. The presentation of the data was initially such that it was not possible to find out cases in which both residues were found in the same sample. The data are available on the internet. When the results of the 2001-2006 plans were evaluated more than 2300 samples analyzed for malachite green residues have been found. The main fish species covered were trout and salmon, including imports. Occasionally the data are scheduled under “imported farmed fish”.

Another useful data set is reported from Denmark. Rasmussen (2007) reported on findings of malachite green in fish in Denmark from 1988 to 2005. 446 plus 95 “targeted samples” were taken. 48 plus 82 “targeted samples” were positive. The author gives individual results for six samples and ranges for the rest of the positives. Unfortunately the individual data were therefore largely not available to the Committee.

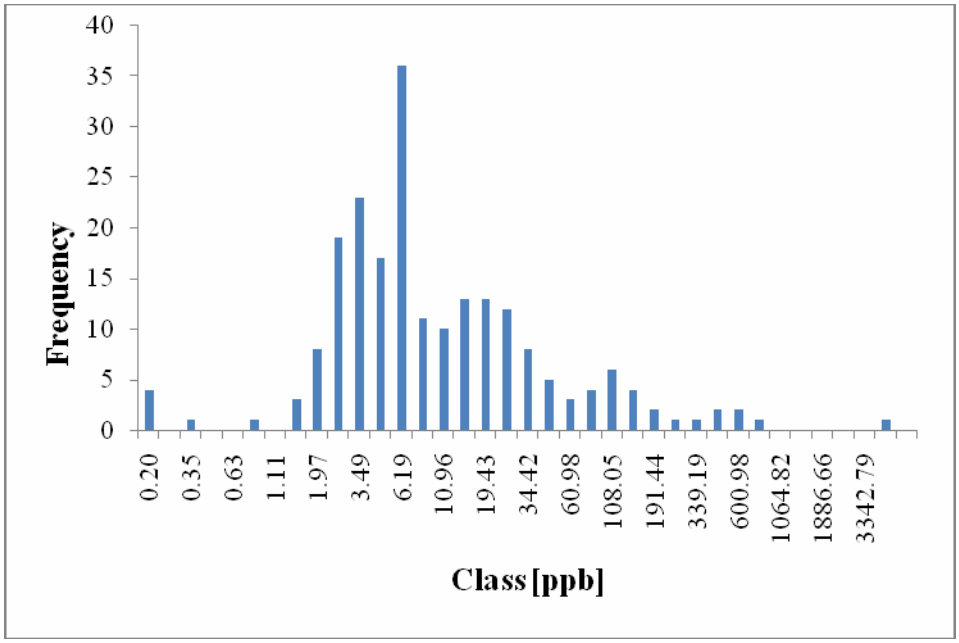
Of 3277 samples selected from these reports, 222 samples were reported positive for malachite green in the range from 0.2 to about 600 µg/kg fish muscle. For many of the samples it cannot be defined what malachite green means (malachite green, leucomalachite green, or the sum of both or just a number because the method was inadequate). Most likely the true concentrations were higher than the results obtained and a significant fraction of the negatives were probably false negatives.

An exhaustive search all public sources of information for data on residues of malachite green is impractical. Many fish species currently moving in international trade and commonly eaten in many parts of the world are not covered. It is not known to what extent results of random sampling and of biased sampling is mixed. It is not known whether any recognised sampling plan has been used in the sampling of lots and which of the individual results given have been obtained from the same lot. The currently published surveillance data are not transparent enough to use it for intake estimates.

The more systematically collected data of the UK from the above described activities can be used to estimate a worst-case figure of upper limit of intake of malachite green resulting from illegal uses. These were monitoring data (spanning from 1995 to 2006) published in the United Kingdom on the occurrence of MG and LMG in fish muscle. If both substances were found in a sample a calculated sum could be determined. The estimated mean level found in the positive samples was 30.7 µg/kg fish muscle and the level at the 97.5th percentile to be 138 µg/kg. Assuming the daily consumption of fish to be 300 g per person, the daily exposure to the sum of malachite green and leucomalachite green can be calculated to be 9.2 and 41 µg/per person at the mean and 97.5th percentile, respectively. For a 60-kg person, this would be equivalent to 0.15 µg/kg bw per day and 0.69 µg/kg bw per day, respectively.

The Figure 8 below shows the frequency distribution of the levels found in the positive samples out of the 3277 samples selected from the above mentioned programs.

Figure 8: Non-representative frequency distribution of illegal residues of malachite green in fish.



The assumption of consumption of 300g of fish contaminated with malachite green and leucomalachite every day for a lifetime was used (a highly conservative assumption). In addition, it was assumed that the concentrations of malachite green and leucomalachite would not change during cooking of the fish.

The study by Law (1994) also was suitable to use for performing a dietary exposure estimate. Such an estimate provides some information on the order of magnitude of likely human exposure to residues of malachite green in case the drug would be authorized for treatments comparable to those performed by the authors.

Depletion half-lives of 28 hours for malachite green and 197 h for leucomalachite green were determined. The kinetic parameters, including the variance of the data, were used to calculate model intakes for every day of 80 years of a human lifespan, assuming daily consumption of 300g of fish muscle. For this purpose 29220 approximately log-normally distributed random numbers were generated for each time point of interest ranging from the predicted value of the regression line minus four times the residual variance to the same predicted value plus four times the residual variance. These calculations were repeated for a number of assumed slaughter times of the fish, ranging from 1h (end of treatment) to 500 h. The results were expressed in mg malachite green/leucomalachite green/kg of human body weight. The minima, maxima and several percentiles, including the median of these estimated daily intakes, were calculated. The median was used for an assessment of chronic intake. The median daily intake of leucomalachite green declined from 7.3 µg/kg bw at hour 1 to 0.87 µg/kg bw at 500 h. Results of an intake assessment for malachite green and leucomalachite green are shown in Table 11 below.

The Committee considered that the assumption of consumption of 300 g of fish contaminated with malachite green and leucomalachite green every day for a lifetime made these estimates highly conservative. In addition, it was assumed that the concentrations of malachite green and leucomalachite green would not change during cooking of the fish. However, that may not be the case.

Mitrowska, et al. (2007) investigated the stability of malachite green and leucomalachite green in muscle of treated carp under various conditions of cooking. The initial concentrations of the residues were approximately 200µg/kg. Leucomalachite green was much more stable than the parent compound. Microwaving was the most effective way to partly destroy the incurred residues. The authors published time curves of the degradation. The end results are summarised in Table 10.

Table 10: Stability of malachite green and leucomalachite green under various conditions of cooking.

Sample	Procedure	Temperature [°C]	Duration [min]	% reduction	
				MG	LMG
Residues in carp muscle	Boiling, baking		15	54	0
	Microwave		1	61	40
Standard solutions	Boiling water	100		0	0
	Cooking oil	150	10	49	
			90	97	
			120		0
		210	10	97	18

Table 11: Results of an intake assessment for malachite green and leucomalachite green.

	Part I – Estimated intake at various theoretical slaughter times of fish														
	1h	**1.6h**	**2.4h**	**3.8h**	**5.9h**	**9.2h**	**14.3h**	**22.4h**	**34.9h**	**54.3h**	**84.7h**	**132.0h**	**205.8h**	**320.8h**	**500.0h**
	Intake of Malachite green [µg/kg bw per day]														
min	0.2	0.2	0.2	0.2	0.2	0.2	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.0
P50	1.8	1.8	1.7	1.7	1.6	1.5	1.3	1.1	0.8	0.5	0.2	0.1	0.0	0.0	0.0
P90	3.8	3.8	3.7	3.6	3.4	3.1	2.8	2.3	1.6	1.0	0.5	0.2	0.0	0.0	0.0
P95	4.7	4.7	4.5	4.4	4.2	3.8	3.4	2.8	2.0	1.3	0.6	0.2	0.0	0.0	0.0
P97.5	5.6	5.6	5.4	5.2	5.0	4.6	4.0	3.3	2.4	1.5	0.7	0.2	0.0	0.0	0.0
P99	6.9	6.9	6.6	6.5	6.1	5.7	5.0	4.1	2.9	1.8	0.9	0.3	0.0	0.0	0.0
max	15.6	16.2	21.1	15.2	14.2	14.1	11.8	9.8	10.4	3.8	2.0	0.6	0.1	0.0	0.0
	Estimated intake of Leucomalachite green [µg/kg bw per day]														
min	1.5	1.4	1.5	1.3	1.5	1.1	1.3	1.2	0.9	0.8	0.8	0.5	0.3	0.3	0.1
P50	7.3	7.3	7.2	7.1	6.9	6.7	6.4	6.0	5.5	4.8	4.0	3.3	2.5	1.7	0.9
P90	12.6	12.6	12.3	12.2	12.0	11.7	11.3	10.7	10.0	8.9	7.8	6.4	4.8	3.3	1.7
P95	14.7	14.7	14.5	14.6	14.1	13.8	13.3	12.6	11.7	10.7	9.3	7.7	5.8	4.0	2.1
P97.5	16.9	17.0	16.6	16.8	16.4	15.8	15.3	14.5	13.5	12.5	11.0	9.1	6.9	4.6	2.4
P99	19.7	19.5	19.6	19.7	19.1	18.9	18.3	17.3	16.0	15.0	13.4	10.9	8.3	5.6	3.0
max	48.5	42.4	36.0	39.7	36.2	34.6	38.0	38.9	31.5	32.0	30.3	22.6	16.6	14.3	6.4
	Estimated intake of the sum of Malachite green and leucomalachite green [µg/kg bw per day]														
min	1.7	1.6	1.7	1.4	1.7	1.3	1.4	1.3	1.0	0.8	0.8	0.5	0.3	0.3	0.1
P50	9.1	9.1	8.9	8.7	8.5	8.2	7.7	7.1	6.3	5.3	4.3	3.4	2.5	1.7	0.9
P90	16.4	16.4	16.0	15.8	15.4	14.8	14.1	12.9	11.6	9.9	8.3	6.6	4.9	3.3	1.7
P95	19.4	19.4	19.0	19.0	18.3	17.6	16.7	15.4	13.8	12.0	9.9	7.9	5.9	4.0	2.1
P97.5	22.5	22.5	22.0	22.0	21.4	20.4	19.3	17.8	15.9	14.0	11.7	9.3	7.0	4.6	2.4
P99	26.7	26.4	26.2	26.3	25.2	24.6	23.3	21.4	18.9	16.8	14.3	11.2	8.4	5.6	3.0
max	64.1	58.6	57.1	54.9	50.4	48.8	49.8	48.7	42.0	35.8	32.3	23.2	16.7	14.3	6.4

Note: For ease of reading and formatting the data, the table entries are rounded values using standard rounding techniques.

Results obtained from other surveys

Only a few examples of the type of information available from surveys can be given here in order to facilitate the discussion of the limited usefulness of such results in the context of intake assessments.

Example 1*:* The Centre for Food Safety of the government of the Hong Kong special administrative region frequently informs consumers about findings of noncompliant foods. In four separate reports from December 2006 to November 2007, 29 positives were reported from 130 samples of varying sea and fresh water samples collected at import and local markets at concentrations of 14 – 480 µg/kg.

Example 2: Reports on monitoring malachite green in aquatic species are available from Australia and New Zealand (FSANZ, 2005). The 60 samples of 7 species of fish were from eight countries of origin. The LOQ for malachite green was 2µg/kg using an LC-MS/MS method. The range of malachite green concentrations was from 4 – 138 µg/kg.

Example 3: The Canadian total diet study (1993-2004) collected shrimp and fish samples of various species from various sources to prepare 30 composite samples for analysis of residues of veterinary drugs (Tittlemier, 2007). Fish were baked at 230 °C for approximately 10 minutes. Shrimp were boiled in tap water. It is unlikely that malachite residues are stable under these conditions of sample preparation. It would have been useful to include composite samples of raw fish. The composite samples were frozen and stored at -20 °C until analysis in 2005. The report makes no statement on the stability of residues over such long storage times at relatively high temperatures. The LC-MS/MS methods used for the determination of malachite green and leucomalachite green had a limit of detection of 0.15 µg/kg. Leucomalachite green was found in three of the compos*ite* samples (freshwater fish 2002 and 2003, 0.95 and 0.73 µg/kg; shrimp 2002, 1.2 µg/kg).

Results obtained from residue depletion studies

Residue depletion studies are only available for the rainbow trout, the channel catfish and tilapia. Only one of all the residue studies discussed above is suitable to predict the time course of residues of malachite green in fish. Table 12 summarises some selected characteristics of four major kinetic and residue studies. Despite the large differences in bath size the ratio of fish weight to bath weight is similar; however, there are large differences in the amounts of drug available per fish. This is most likely a critical factor in studies with prolonged exposure times. The figure is lowest for the study with the longest exposure time.

Only one study, conducted by Law (1994), replaced the malachite green taken up from the bath by the fish. The main argument against using the data of the Alderman and Clifton-Hadley study (1993) is that the otherwise well designed study exhibited methodological deficits and used an analytical method that was not valid for the purpose. The Plakas, et al. (1996) study is excellently designed and conducted, but the individual fish residue data were not available. The Bauer, et al. study could have been well used for an observation period similar to the exposure time in the other study; however, individual data were also not available. Overall, only limited conclusions are possible for this study because most of the malachite green was used up by the fish during exposure to the bath.

Table 13 highlights some possible impact on results of bath treatment studies regarding the study designs. For the calculations in the table the physiological data for trout discussed in a previous section were used. Three options regarding ventilation volume and breaths/min were calculated for the Alderman and Clifton-Hadley study (1993), one alternative is given for the Bauer, et al. study (1988). From the data presented the design of the Alderman and Clifton-Hadley study was largely acceptable, however, it was not the case for the Bauer et al. study. This is further substantiated in Figure 10.

Table 12: Selected characteristics of four kinetic residue studies.

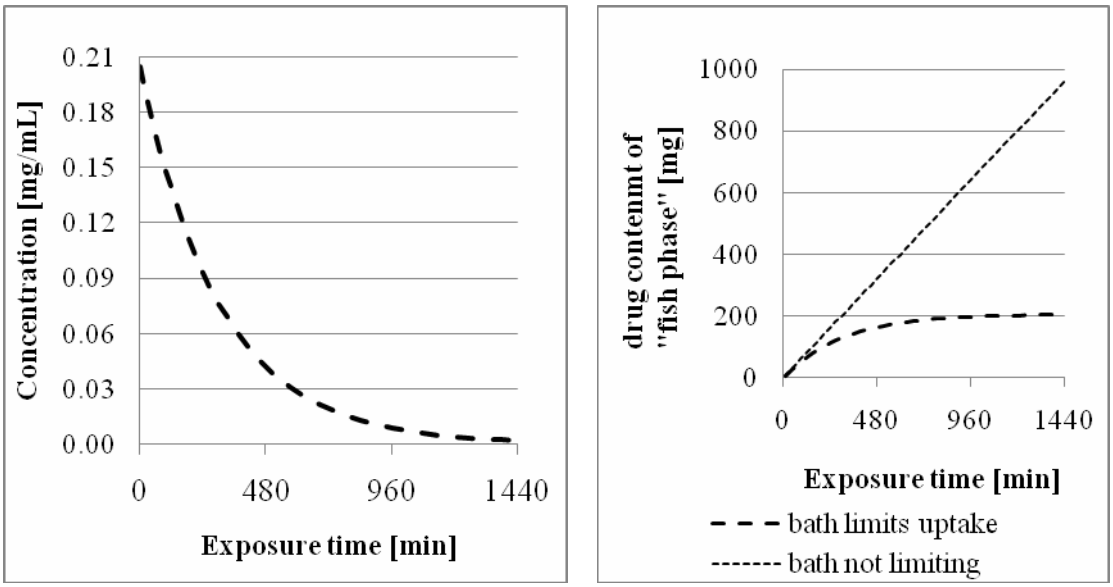
Authors and year	Species	Volume of the tank used for treatment [L]	Initial concentration of malachite green [mg/L]	Average temperature [° C]	Duration of exposure [min]	Estimated maximum weight of all fish in the tank	Ratio bath weight to fish weight	Initial amount of drug available [of fish]
Bauer, et al., 1988	Rainbow trout	1000	0.21	9.7	1440	41.2	24.27	4.98
Alderman and Clifton-Hadley, 1993	Rainbow trout	725	1.6	16 8	40	28.92	25.07	40.11
Plakas, et al., 1996	Channel catfish	100	0.8	21	60	3.5	28.57	22.86
Law, 1994	Rainbow trout	200	2.0		60	8.4	23.8	47.6

Table 13: Fish-physiological aspects of selected kinetic residue studies.

Ventilation volume mL/min	Stroke volume [mL]	Breaths/min	Initial MG concentration [mg/L]	Tank volume [L]	Total amount of MG in tank [mg]	Exposure time [min]	Total number of breaths per animal	Number of fish	Stroke volume of all fish together [mL]	Total inspired water during exposure [L]
22	0.5	44	1.6	725	1160	40	1760	120	60	105.6
37	0.5	74	1.6	725	1160	40	2960	120	60	177.6
49	0.5	98	1.6	725	1160	40	3920	120	60	235.2
22	0.5	44	0.205	1000	205	1440	63360	150	75	4752

For a primitive modelling exercise it was assumed that the inspired water is completely cleared from malachite green which means the amounts inspired with a stroke remain in the fish. On this basis the following two graphs (Figure 10) were prepared modelling the situation of the Bauer, et al. study. The data were generated by dissecting the whole uptake process into the number of elementary steps dictated by the above given total number of breaths per animal.

Figure 10: Modelling of some aspects of the Bauer et al. (1988) study.



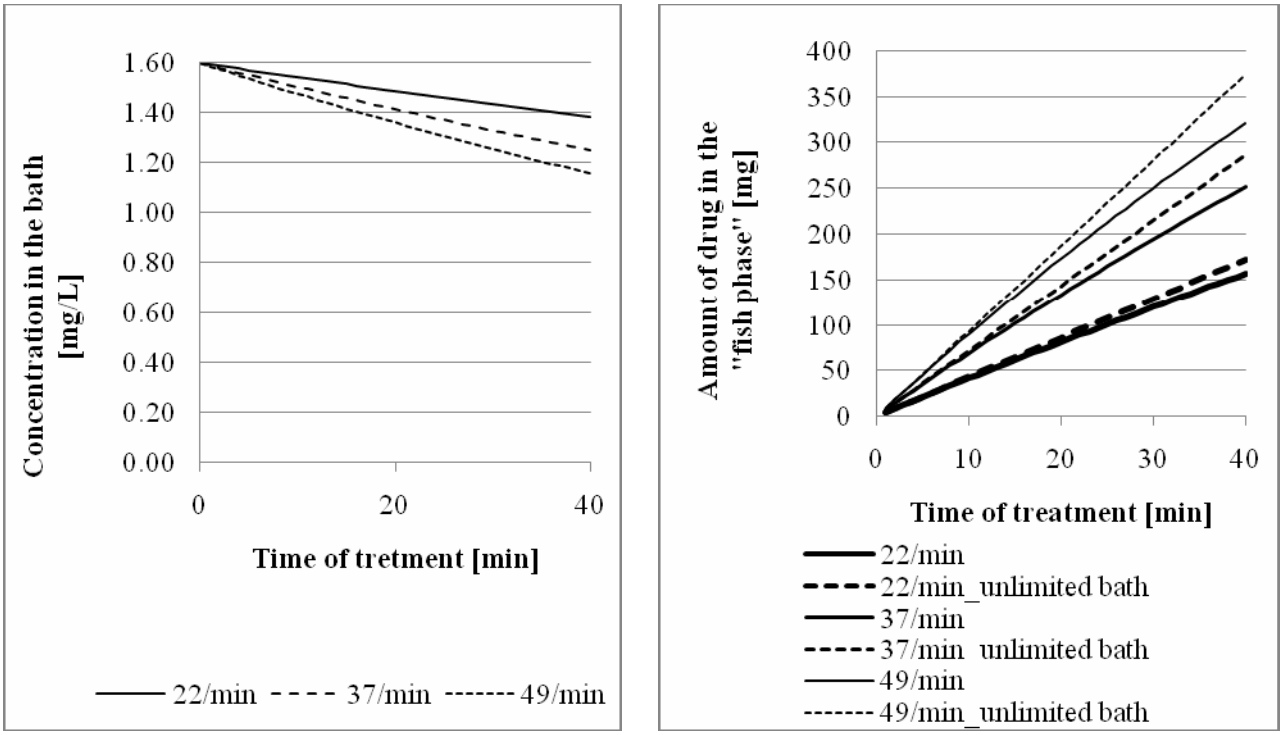
Left side: changes in the drug concentration in the bath; right side: uptake during exposure in a limited bath volume and limited amounts of available malachite green.

The modelling on the left side of Figure 10 predicts that the bath volume is insufficient for an exposure experiment of 1440 minutes duration involving 150 fish. The right modelling experiment predicts that the uptake of malachite green by the fish will be limited by the amount of available drug and therefore, could lead to the wrong interpretation that the uptake capacities of the fish were saturated. The model predicts a final concentration of 2 mg/m^3; the authors reported 5 mg/m^3. Also the predicted amounts of residues in the fish are in the same order of magnitude as experimentally determined by the authors. Although the study has provided some remarkable results it is not representative for the treatment of rainbow trout at 0.8 mg/l in a bath.

Similar calculations were performed using the information from the Alderman and Clifton-Hadley study (1993) and are summarized in Figure 11 below. The graphs show that the concentration in the water bath decreased by approximately 25% in the worst case model using the highest ventilation volume. If one compares the predicted amounts taken up by the fish it appears that the scenario with the lower ventilation volume fits better to the order of magnitude of initial tissue residue concentrations given by the authors. Thus the study was well designed but suffered from a number of weaknesses discussed in a previous section.

It was not possible to perform similar modelling with the information provided in the Plakas et al. study (1996). However, this was also not necessary because the authors were prudent to use a fresh bath for every set of five fish treated and they report that the concentration of malachite green in the bath decreased by only approximately 15% during treatment.

Figure 11: Modelling of some aspects of the Alderman and Clifton-Hadley study (1993).



Left side: Changes in bath concentration as function of strokes/min; Right side: Changes in drug uptake as function of strokes/min.

METHODS OF ANALYSIS FOR RESIDUES IN TISSUES

Sample preparation

Animal tissues

Typically, residues of malachite green and leucomalachite green are extracted from (2 - 5 g) homogenized animal (catfish, eel, rainbow trout, salmon, tropical prawn, turbot, carp, tilapia, tiger shrimp) tissues (raw or cooked muscle samples). Amber flasks are generally used in the protocol to avoid photo-degradation phenomena that would occur during sample preparation.

The typical protocols for malachite green and leucomalachite green extraction involve vortex mixing or shaking in acetonitrile mixtures (extraction is generally performed over 3 to 15 minutes from 500 to 4000 rpm); the inclusion of anti-reductants and radical scavengers has been common practices. Several acetonitrile buffer extractions protocols are reported: mixture of McIlvaine buffer (pH 3.0, 18.9 ml 0.2 *M* sodium hydrogen phosphate and 81.1 ml 0.1 *M* citric acid) and 12 ml acetonitrile (Bergwerff and Scherpenisse, 2003; Dowling, et al., 2007); acidic (0.1% acetic acid) acetonitrile with NaCl (Hernando, et al., 2006); 0.1M ammonium acetate pH 4.5 and acetonitrile (Andersen, et al., 2005, 2006; Tarbin et al., 2008; Hall, et al., 2008). Other mixtures such as hydroxylamine solution (25%), 0.5 ml of *p*-toluenesulfonic acid solution (1 M) and 5ml of acetate buffer (0.05 M, pH 4.5) are also reported (Mitrowska, et al., 2005; Mitrowska, et al., 2007; Andersen, et al., 2008).

Purification is generally performed with SPE and /or liquid/liquid extraction with dichloromethane. Clean up over SPE may be carried out over aromatic sulfonic acid solid-phase extraction columns (Bergwerff and Scherpenisse, 2003; Anderssen, et al., 2008). Malachite green and leucomalachite green are eluted with the following mixture: 2.5 ml 1.0 mg/ml methanolic ascorbic acid, 20 ml 50 mM sodium perchlorate containing 25 mM sodium acetate and 25 mM 1-pentanesulfonic acid adjusted to

pH 4.0 with acetic acid, and 27.5 ml acetonitrile (Bergwerff and Scherpenisse, 2003). Other elution conditions have been reported on the same SPE cartridges: 90% (v/v) methanol, 5% (v/v) of 1mg/ml ascorbic acid and 5% (v/v) of 25% (m/v) aqueous NH_4OH (Scherpenisse and Bergwerff, 2005). Purification is also reported on Strata SCX (strong cation-exchange) disposable columns with a mixture containing acetonitrile and ammonium hydroxide (25%) (90/10) (Mitrowska, et al., 2005; Tarbin, et al., 2008) or with citrate buffer/acetonitrile (Stubbings, et al., 2005).

Few papers report the development of Molecularly Imprinted Polymers (MIP) based SPE for selective purification of malachite green from fish water and fish feed samples. Malachite green is used as template, methacrylic acid (MAA) as the functional monomer and ethylene glycol dimethacrylate (EGDMA) as the linking agent (Li, et al., 2008). Eighty percent cross reactivity with leucomalachite green was observed. The use of malachite green as a template might however lead to a bleeding phenomenon.

Solid-Liquid (SLE) extraction methods are also reported for the purification step using Bondesil-NH2, 40 µm particle size (Hernando, et al., 2006).

The literature also reports some protocols with an "*in situ*" quantitative oxidation of leucomalachite green into MG by reaction with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone after the acetonitrile extraction step. Resulting total malachite green is then subsequently purified by solid phase extraction with alumina and propylsulfonic acid phases (Anderssen, et al., 2005, 2006).

HPLC analysis - Screening tests

Current methods for the determination of malachite green and leucomalachite green in fish tissues or water are based on liquid chromatography (LC), mainly with visible (VIS)/fluorescence (FLD) on-line detections. The parent compound has λ_{max} at 620 nm, whereas the leuco form has λ_{max} at 265 nm, making it difficult to determine malachite green and the leuco form using the same conditions. In practice, the absorbance detector is set at 620 nm (Bergwerff and Scherpenisse, 2003; Mitrowska, et al., 2005, 2007, 2008; Anderssen, et al., 2008) or 618 nm (Anderssen, et al., 2005; Stubbings, et al., 2005) for malachite green detection while the fluorescence detector is set at λ_{ex} = 265 nm and λ_{em} = 360 nm for leucomalachite green detection (Mitrowska, et al., 2005, 2007, 2008; Anderssen, et al., 2008).

Chromatographic separation is reported on phenyl-hexyl analytical columns fitted with corresponding guard columns; the mobile phase consisting in acetonitrile and acetate buffer (0.05 M, pH 4.5) (70:30, v/v) in isocratic conditions (Mitrowska, et al., 2005, 2007, 2008). The use of reversed-phase analytical chromatographic columns is also reported in this context - ODS-2 (Bergwerff and Scherpenisse, 2003), Alltima C18 (Anderssen, et al., 2005, 2008) with acetonitrile based mobile phases such as mixture of sodium perchlorate containing pentanesulfonic acid and acetonitrile in a ratio 2:3 (v/v) (Bergwerff and Scherpenisse, 2003) or ammonium acetate buffer/acetonitrile 50/50 (v/v) (Anderssen, et al., 2005, 2008, Stubbings, et al., 2008).

Simultaneous LC-VIS determination of both forms is possible by post-column oxidation of leucomalachite green to malachite green to convert the colorless leuco form into the chromophore using cartridge containing lead(IV) oxide (PbO_2) (Allen and Meinertz, 1991; Allen, et al., 1992; Swarbrick, et al., 1997; Rushing. et al., 1995; Tarbin, et al., 1998; Bergwerff and Scherpenisse, 2003). Post column oxidation protocols are also reported (Valle, et al., 2005). Electrochemical oxidation has been used as an alternative to PbO_2 (Rushing, et al., 1997). The determination of both compounds together constitutes a good screening method to confirm the presence of this kind of residue, taking into account that the combined signals will provide a gain of sensitivity. Detection limits reported for LC-VIS measurements are around 1 µg/kg.

More recently, screening tests involving mass spectrometric detection have been reported for the simultaneous measurement of malachite green and leucomalachite green. The sum of malachite green

and leucomalachite green is determined by liquid chromatography coupled to atmospheric pressure chemical ionisation mass spectrometry (LC-APCI-MS) after post column oxidation (Valle, et al., 2005). Detection limit obtained on spiked salmon samples based on ion at m/z 313 is 0.15 µg/kg. Typical recoveries are in the range 70-85%.

Screening tests involving Surface-Enhanced Raman microfluidic sensors have also been reported for water analysis. This kind of biosensor allows fast and sensitive trace analysis of malachite green (Lee, et al., 2007; Lucotti, et al., 2007). Malachite green molecules are adsorbed onto silver nanoparticles while flowing along the polydimethylsulfoxane (PDMS) channel. A quantitative analysis of malachite green is performed based on the measured peak height at 1615 cm^{-1} in its SERS spectrum. Corresponding limit of detection was found around 1-2 µg/kg.

Finally, ELISA tests have also been developed for selective detection of malachite green and the related triphenylmethane dyes in fish and fishpond water (LOD = 0.05 µg/L in water) (Yang, et al., 2007). Performance characteristics are noted below.

LOQ: 0.49 µg/kg (malachite green, UV-VIS) (Andersen, et al., 2008)
LOD: 0.15 µg/kg (MS detection ion m/z 313 (MG+LMG)), 0.15 µg/kg (MG, UV-VIS, (Andersen, et al., 2008); 1 µg/kg (MG + LMG, UV-VIS) (Bergwerff and Scherpenisse, 2003, Andersen, et al., 2006); 1-2 µg/kg (SERS) (Lee, et al., 2007).
CCα: 0.15 µg/kg (MG UV-VIS), 0.13 µg/kg (LMG, FLD) (Mitrowska, et al., 2006)
CCβ: 0.37 µg/kg (MG UV-VIS), 0.32 µg/kg (LMG, FLD) (Mitrowska, et al., 2006)
Linearity: R^2 =>0.995
Precision: RSD 7.7-10.9% (MG, UV-VIS) 7.7-8.4%(LMG, FLD) (Mitrowska, et al., 2006)
Accuracy: 60-64% (MG, UV-VIS), 89-92% (LMG, FLD) (Mitrowska, et al., 2006)
Recovery: 85-90% LMG (Andersen, et al., 2008; Mitrowska, et al. 2005), 60-70% MG (Andersen, et al., 2008, Mitrowska, et al. 2005)

Confirmatory methods

For confirmatory purposes analytical procedures utilize detection by mass spectrometry (MS) with liquid or gas chromatography, which does not demand post-column oxidation of leucomalachite green (Turnipseed, et al., 1995a). However, either the PbO_2 reactor or the "*in situ*" oxidations are used with MS, because detection of malachite green is more sensitive comparing it with leucomalachite green (Tarbin, et al., 1998; Bergwerff and Scherpenisse, 2003).

Gas chromatography coupled to mass spectrometry (GC-MS) analysis

GC-MS analyses were first developed in the mid 1990's to provide confirmatory methods for leucomalachite green in fish tissues (Turnipseed, et al., 1995b). Selected ion monitoring was performed based on five diagnostic ions (m/z 330, 329, 253, 210 and 165).

Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) analysis

Chromatographic separation is generally performed on phenyl phases using either a gradient of acidic acetonitrile (0.1% FA)/ water or an isocratic mixture of acetonitrile/acetate buffer (70/30, v/v) as mobile phases (Andersen, et al., 2006, 2008, Mitrowska, et al., 2008). C_{18} phases with 50mM ammonium acetate/acetonitrile or acidic water/acetonitrile as eluents have also been reported (Bergwerff and Scherpenisse, 2003; Scherpenisse and Bergwerff, 2005; Hernando, et al., 2006, Tarbin, et al., 2008).

Atmospheric pressure chemical ionization coupled to ion trap (APCI-IT)

Atmospheric pressure chemical ionization coupled to ion trap has shown to be a very sensitive and selective technique for the analysis of malachite green which is recovered under $[M]^+$ charged species

with a molecular ion at m/z 329 (Valle, et al., 2005). The use of ion trap as mass analyzer is reported since it allows for full collection of product scan data, further increasing the analytical selectivity of the method (Doerge, et al., 1998b). (MS acquisition program = MS^2 scan of m/z 329, width 2 amu, relative. collision energy 48-50%, activation Q = 0.25, activation time = 30 ms, mass range 150-350) (Andersen, et al., 2006, 2008). The product ions include m/z 314 (M^+-CH_3), m/z 313 (M^+-H-CH_3), m/z 285 (M^+-NC_2H_6), m/z 251 (M^+-C_2H_6), m/z 237 (M^+-C_6H_5-CH_3) and m/z 208 (M^+-C_6H_5 NC_2H_6). High collision energy is needed to obtain significant abundance of these ions.

LC-ESI+-QqQ

Analysis with liquid chromatography coupled to an electrospray ionization-triple quadrupole mass spectrometer allows monitoring malachite green as $[M]^+$ with the following transitions: 329.3>165.0, 329.3>208.0, 329.3>313.3 with associated collision energy ranging from 45 to 75 V; leucomalachite green ($[M+H]^+$ is monitored using the following transitions: 331>239 and 331>316 (Bergwerff and Scherpenisse, 2003, Dowling, et al., 2007; Scherpenisse and Bergwerff, 2005; Mitrowska, et al., 2008; Tarbin, et al., 2008).

LC-ESI+-TOF

Applications have also been reported with liquid chromatography coupled to time-of-flight mass spectrometry with electrospray ionization (R = 9500 FWHM) for improved selectivity, especially with regard to matrix effect (see figure 15) (Hernando et al., 2006). However, sensitivity performances of the corresponding method are not compliant with the legislation since the minimum residue performance level (MRPL) is exceeded.

LC-ESI+-LTQ

One application is reported with a linear ion trap as mass analyzer exhibiting similar performances as observed with ion trap or triple quadrupole technologies (Wu, et al., 2007).

Isotopic internal standards (d_5-MG and $^{13}C_6$-LMG) are available and have been used to overcome problems such as matrix suppression during electrospray ionisation (Hall, et al., 2008)

Performance characteristics of confirmatory methods

LOQ: 0.75 µg/kg (Andersen, et al., 2008)[6 ppb (MG), 3 µg/kg (LMG), TOF (Hernando, et al., 2006)]
LOD: 0.2-0.25 µg/kg (Bergwerff and Scherpenisse, 2003, Andersen, et al., 2006, 2008) [2 µg/kg (MG), 1 µg/kg (LMG), TOF (Hernando, et al., 2006)]
CCα: 0.07-0.14 µg/kg (MG), 0.05-0.17 µg/kg (LMG) (Scherpenisse and Bergwerff, 2005; Dowling, 2007) [8 µg/kg (MG), 38 µg/kg (LMG) TOF (Hernando, et al., 2006)][1.2 µg/kg (MG) multiresidue (Tarbin, et al., 2008)]
CCβ: 0.15-0.23 µg/kg (MG),0.08-0.21 µg/kg (LMG) (Scherpenisse, 2005) [13 µg/kg (MG), 65 µg/kg (LMG), TOF (Hernando, et al., 2006)] [2.0 µg/kg (MG) multiresidue (Tarbin, et al., 2008)]
Linearity: $R^2 > 0.995$ in the range 0.5 – 10 µg/kg (Andersen, et al., 2008)
Accuracy: RSD 10%
Recovery: 85-100%

Extraction and Quantification in incurred samples

Although the ability to detect malachite green and leucomalachite green at regulated levels has been dramatically improved by the use of LC-MS/MS and SPE clean-up procedures, the analysis of malachite green in fish tissues remains a challenge, essentially due to issues surrounding extraction and analyte stability. This issue has been reported recently in literature with a high accuracy method

for quantification of malachite green and leucomalachite green in salmon using exact matching isotope dilution mass spectrometry associated to longer extraction time (16h) (Hall, et al., 2008). Results showed that whilst the total extraction and equilibrium of leucomalachite green was achieved in less than 1h, further malachite green could still be extracted up to 16h. This highlights the difference in chemical behaviour of the two analytes in fish matrix and the necessity for longer extraction time. Further work could concentrate on improving the rate of release of malachite green from fish tissue (e.g., enzymatic digestion). In particular the binding of malachite green to proteins might be an issue in extraction efficiency.

Stability of the analytes in incurred samples

Degradation is reported as less than 10% after 12 months storage at -20°C, however, dramatic degradation is observed for malachite green at room temperature (recoveries from 80 to 40 % in 2 hours), and little effects are observed on leucomalachite green. Malachite green and leucomalachite green recoveries are strongly affected by freeze-thawing cycles and storages at +4°C and -20°C.

Degradation products

The metabolite leucomalachite green is not the endpoint of malachite green transformation and the MRPL fixed at 2 µg/kg and the corresponding sum MG+LMG is an underestimate of the actual presence of malachite green residues. Indeed, several studies have shown that malachite green and leucomalachite green are de-methylated by systematic sequential oxidations (Culp, et al., 1999). (Bergwerff and Scherpenisse, 2008) provide a tabular summary of the structures of residues of malachite green identified in treated rainbow trout.

The degradations products may be formed in living fish organisms during enzymatic action but also during photo-oxidative degradation in water (Mitrowska, et al., 2008; Bergwerff and Scherpenisse, 2008). Some identified degradation products in incurred rainbow trout or in water are shown in figure 16 [m/z 315 (N-demethyl-MG), m/z 301 (N,N-didemethyl-MG); m/z 317 (N-demethyl-LMG); m/z 303 (N,N-didemethyl-LMG)] (Mitrowska, et al., 2008; Bergrwerff and Scherpenisse, 2008). In rainbow trout, these derivatives were found to represent about 20% of the total concentration of malachite green residues. Since these demethyl derivatives are also expected, like malachite green and leucomalachite green, to react with DNA, being thus potential carcinogens, the MRPL (2 µg/kg for MG + LMG) (European Commission Decision 2004/25/EC) may therefore be subjected to future revision.

Recent literature indicates that malachite green undergoes three main photolytic degradations under natural sunlight irradiation: N-demethylation, hydroxylation and cleavage of the conjugated structure forming benzophenone derivatives (Perez-Estrada, et al., 2008). More than 20 transformation products have thus been identified. These processes involve hydroxyl radical attack on the phenyl ring, the N,N-dimethylamine group and the central carbon atom. The *Vibrio fischeri* acute toxicity test showed that the solution remains toxic after malachite green has completely disappeared. This toxicity could be assigned, at least in part, to the formation of 4-(dimethylamine)benzophenone (D20), which is considered 'very toxic to aquatic organisms' by current EU legislation.

Degradation of malachite green and leucomalachite green reported and explained by photo-oxidative demethylation, might be prevented or at least reduced during sample preparation by addition of ascorbic acid (Mitrowska, et al., 2005, 2008) or N,N,N',N'-tetramethyl-1,4-phenylenediamine dihydrochloride (TMPD) to the analytical matrix (Bergwerff and Scherpenisse, 2008).

Multi-residue method sensitivity

As multi-residue protocols are developed and more reported as a trend in scientific literature (Tarbin, et al., 2008), performances (CCα = 1.2 µg/kg and CCβ = 2 µg/kg for malachite green in fish tissues) they may not be compliant, in particular with the MRPL (2 µg/kg MG + LMG).

Influence of processing

Effects of various cooking methods (boiling, baking, microwaving) on malachite green and leucomalachite green have been investigated in incurred carp muscles as noted previously (Mitrowska, et al., 2007). A decrease in concentration was observed for malachite green: - 54% after 15 min boiling or baking; leucomalachite green was stable under these conditions. Microwaving induced a loss of both malachite green and leucomalachite green after 1 min (- 61% and - 40%, resp.). Malachite green also appeared to be degraded in cooking oil at 150 °C (50 % in 10 min).

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MONENSIN

First draft prepared by
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IDENTITY

International Non-proprietary names (INN): Monensin sodium

Synonyms: Monensin A sodium salt; Monensin sodium; Monensin sodium salt; NSC 343257; Sodium monensin; Elancoban®; Elancogran®, Coban®, Rumensin®, Coxidin®

International Union of Pure and Applied Chemistry (IUPAC) Names: Stereoisomer of 2-[2-ethyloctahydro-3′methyl-5′[tetrahydro-6-hydroxy-6-(hydroxymethyl)]-3,5-dimethyl-2H-pyran-2-yl] [2,2′-bifuran′5′yl]]-9-hydroxy-β-methoxy-a,γ,2, 8,-tetramethyl-1,6-dioxaspiro[4.5]decan-7-butanoic acid.

And: 4-[2-[5-ethyl-5-[5-[6-hydroxy-6-(hydroxymethyl)-3,5-dimethyl-oxan-2-yl]-3-methyl-oxolan-2-yl]oxolan-2-yl]-9-hydroxy-2,8-dimethyl-1,6-dioxaspiro[4.5]dec-7-yl]-3-methoxy-2-methyl-pentanoic acid;

Chemical Abstract Service (CAS) Number: Monensin 17090-78-8
Monensin Sodium 22373-78-0

Structural formula of main components:

Figure 1:

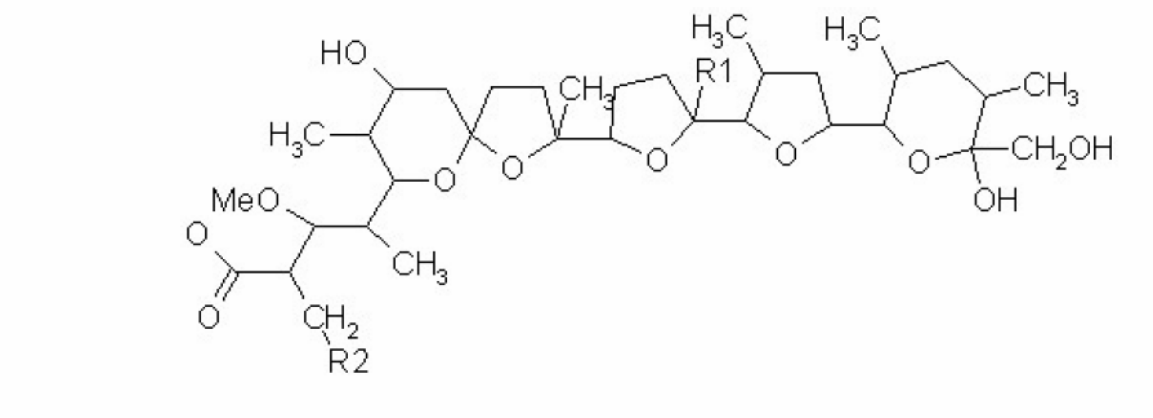

Table 1: Summary of Monensin Factors.

Monensin Factor	R_1	R_2	R_3
A	C_2H_5	H	H
B†	CH_3	H	H
C*	C_2H_5	H	CH_3
D*	Not supplied		

†Factor B accounts for less than 4% of the total composition.
*The relative biological activity of trace factors C and D assayed against *Streptococcus faecium* are negligible (Haney and Hoehn., 1967; Agtarap and Chamberlin, 1967; Chamberlin and Agtarap, 1970).

Molecular formula: Monensin A (sodium salt): $C_{36}H_{61}O_{11}Na$
Monensin B (sodium salt): $C_{35}H_{59}O_{11}Na$

Molecular weight: Monensin A (sodium salt): 692
Monensin B (sodium salt): 678

OTHER INFORMATION ON IDENTITY AND PROPERTIES

Pure active ingredient:

Appearance: Off-white to tan crystalline powder

Melting point: 267-269˚ C (sodium salt); 103-106° C (acid)

Solubility: Soluble in ethyl acetate, acetone, chloroform and dimethyl sulphoxide. Essentially insoluble in water and petroleum ether.

RESIDUES IN FOOD AND THEIR EVALUATION

Conditions of use

Monensin sodium is used for the control of coccidiosis in chickens, turkeys, and quail. In feedlot and pasture cattle, it is used to improve the efficiency of rumen fermentation, increase rate of weight gain and for the prevention and control of coccidiosis. In feedlot and lactating and non-lactating dairy cattle, it is used to control ketosis. In dry and lactating dairy cows, it is used to increase milk production efficiency (production of marketable solids-corrected milk per unit of feed intake). In calves, non-lactating goats, and sheep it is used for prevention and control of coccidiosis.

Dosage

Monensin sodium is provided in a complete feed at maximum use concentrations of 125 mg/kg feed for broiler chickens and 120 mg/kg feed for replacement layers. The maximum dose for turkeys is 100 mg/kg feed; for quail, the maximum dose is 73mg/kg feed.

To improve the efficiency of rumen fermentation, monensin sodium is provided at a maximum dose of 360 mg/animal/day or 40 mg/kg in complete feed for feedlot cattle and 200 mg/animal/day in a 0.45 kg feed supplement for pasture cattle. For control of ketosis in feedlot cattle, the maximum dose is 480 mg/animal/day. To control ketosis in lactating dairy

cattle, monensin is administered as a controlled-release capsule providing a maximum daily dose of 400 mg/animal, released into the rumen. It may be given to dry and lactating dairy cows continuously as a total mixed ration containing 11 to 22 g monensin/ton (12 to 24 mg/kg). In calves, it is provided at a maximum dose of 200 mg/animal/day. In the USA, it is explicitly labelled not to be used in calves raised for veal. In non-lactating goats, it is provided as the sole ration containing 20 g monensin/ton (22 mg/kg). In sheep, it is provided in the total ration at a rate of not less than 11 and not more than 22 g monensin/metric ton.

PHARMACOKINETICS AND METABOLISM

Pharmacokinetics in Laboratory Animals

Rats

The disposition of orally administered [^{14}C] monensin was determined in rats treated for 13 days with feed containing 100 mg unlabelled monensin/kg feed. On day 14, rats received a single oral dose of radiolabelled monensin, 2.15 mg (specific activity 0.0266 μCi/mg), by gavage. Thereafter, for the remainder of the study (12 days), rats again received unlabelled monensin in the feed. Unlabelled feed was provided *ad libitum*. Urine and faeces were assayed daily for radioactivity. Within three days after dosing with the labelled drug, 92.0% of the total radioactivity was recovered. The majority of the radioactivity was recovered in the faeces (91.5%) but a small amount was recovered in urine (0.5%). Selected tissues and organs were assayed for radioactivity but the detected radioactivity was not different from that measured in pooled control tissues (Herberg, 1973a). Monensin and monensin metabolites were isolated from the liver and faeces of male and female rats treated orally with 5 mg [^{14}C] monensin/kg body weight (Donoho, 1985).

In another study (Howard and Lobb, 1981), tissue distribution and biliary elimination of radioactivity was evaluated in male and female rats following oral administration of [^{14}C] monensin. Radiolabelled monensin (0.614 μCi/mg) was administered orally at doses ranging from 5 to 40 mg/kg body weight in male rats and 2 to 16 mg/kg body weight in female rats. The doses were based on previously determined oral toxicity data (Broddle and Worth, 1976) for male (40.1±3.0 mg/kg) and female (24.3±2.7 mg/kg) rats. After administration, radioactivity was eliminated rapidly and extensively in faeces during a 72-hour collection period. Faecal elimination accounted for 83.6-87.4% and 70.8-87.2% of the dose in male and female rats, respectively. Urinary excretion represented only a minor route of elimination representing 1.0-1.6% and 1.0-1.3% of the dose in male and female rats, respectively. Biliary secretion was the primary excretory pathway following oral administration in rats. There were no differences in the urinary, faecal, or biliary excretions of radioactivity between male and female rats. At toxic doses, there was an initial delay in the excretion of radioactivity in faeces and bile considered secondary to the toxicity. Further support for partial gastrointestinal absorption of monensin in male and female rats was found in studies that showed that 31 to 53% of a radioactive dose (32.8-46.6% in males and 30.7-53.2% in females) of 2 to 40 mg/kg body weight was collected in bile within 72 hours of administration (Howard and Lobb, 1981).

Pharmacokinetics in Food Animals

Cattle

Gastrointestinal absorption of monensin in cattle has been evaluated. In one study, absorption of [^{14}C] monensin in calves was evaluated by measuring radiolabelled residues in bile (Davison, 1984). The amount of radiolabelled material recovered in bile can serve as an estimate of the amount of material absorbed because little monensin is excreted in cattle urine. Two calves, one male and one female, were fitted with bile duct cannulae. Each calf

received a single oral dose of 10 mg [^{14}C] monensin/kg body weight in a gelatine capsule. Bile was collected continuously for 72h. Approximately 35 and 37% of the administered radioactivity was recovered in the bile from the male and female calf, respectively. The presence of monensin or monensin metabolites in plasma (Donoho, 1984), liver and milk (Herberg, et al., 1978; Kline and Wicker, 1975; Kennington, et al., 1995) from orally treated animals provides supporting evidence for absorption.

Chickens and Turkeys

The pharmacokinetic profile of monensin was evaluated in broiler chickens (Atef, et al., 1993) following administration by gavage and intravenously as a single dose of 40 mg/kg body weight. Following intravenous administration, disposition of monensin followed a two-compartment open model. The absorption half-life was 0.6 hours, the volume of distribution was 4.1 L/kg, and the total body clearance was 28.4±0.2 ml/kg/min. The highest serum concentration (4.1±0.05 μg/ml) was reached after 0.4 hours following administration by gavage. The absorption half-life was 0.3 hours and the elimination half-life was 2.1 hours. A somewhat longer terminal elimination half-life (3.1 to 5.6 hours) has been determined recently (Henri, et al, 2008a). *In vitro* serum protein binding was calculated to be 22.8%. Bioavailability following administration by gavage was 65.1% (Atef, et al., 1993). In chickens, monensin concentrations in serum and tissues were higher after administration by gavage (40 mg/kg body weight) than after feeding a diet containing 120 mg monensin/kg for 2 weeks (average daily consumption was 24 mg monensin based on a daily feed consumption of 200 g of medicated feed).

The rate of faecal excretion and quantitation of orally administered monensin in chickens were determined. Chickens received an oral dose of [^{14}C] monensin (7.36 mg, specific activity 0.018 μCi/mg). Seventy-five percent of the administered dose was eliminated in excreta within 3 days and was eliminated completely within 12 days (Herberg, 1973b). In another study, three chickens were treated *ad libitum* with feed containing 120 mg unlabelled monensin/kg feed. Chickens were then dosed by oral capsule with a single dose of [^{14}C] monensin. More than 75% of the radioactivity was recovered within 3 days following the dose. Radioactivity in excreta returned to background levels in 4, 5, and 12 days (Herberg, 1975a). In another study (Grundy, *et al.*, 1998), chickens were fed a ration containing 125 mg [^{14}C] monensin/kg feed for 6 days then slaughtered 6h, 1, 3 or 5 days after the treated feed was withdrawn (3 male and 3 female chickens per withdrawal group). Bile contained approximately 87mg monensin/kg after 6h withdrawal. By 5 days withdrawal, bile contained approximately 0.4mg monensin/kg and approximately 76% of the dose had been recovered in excreta. These data indicate that radiolabelled monensin is eliminated rapidly and quantitatively by chickens.

In turkeys, the evidence of intestinal absorption is analogous to that for chickens. Monensin and metabolites were found in turkey liver at zero withdrawal following *ad libitum* access feeding with 110 mg [^{14}C] monensin/kg feed for five days (Donoho, et al., 1982a). The reported terminal elimination half-life in turkeys is 1.4 to 1.6 hours (Henri, et al, unpublished 2008a).

Metabolism in Laboratory Animals and Humans

Rats

Data from studies in laboratory animals indicate that monensin is extensively metabolized prior to excretion. The *in vitro* metabolism of monensin has been evaluated in several studies (Ershov, et al., 2001; Nebbia, et al., 1999; Nebbia, et al., 2001). In liver microsomes induced by dexamethasone, monensin is metabolized by the 3A family of cytochrome P-450. Inducers of O-demethylation enhance and inhibitors reduced monensin metabolism. Although

monensin is a substrate for P-450, it does not appear to be a direct *in vitro* inhibitor of rat liver microsomes (Ceppa, et al., 1997). Studies concluded that for drugs commonly administered to humans, monensin is unlikely to inhibit human P-450 directly and would not affect drug metabolism attributable to this family of enzymes (Ueng, et al., 1997). However, it is hypothesized that drugs that inhibit P-450 enzymes could result in toxic interactions with monensin, including drugs potentially administered concurrently with the ionophore (tiamulin or several macrolides) (Nebbia, et al., 1999). Metabolites M-1, M-2, M-3, M-6, and M-7 have been identified in liver and/or excreta from monensin-exposed rats (Donoho, 1985).

Mice

No data were provided on metabolism in mice.

Dogs

The *in vitro* metabolism of monensin also has been evaluated in microsomal incubates from dogs using monensin concentrations of 0.5, 1.0, and 10 µg/mL. The microsomes were sourced from pooled samples comprising more than one donor. An HPLC/MS with electrospray ionisation was used to measure the disappearance of parent drug (monensin A) at multiple time points following incubation. Data indicate that monensin is metabolized by first order kinetics in all cases, consistent with a metabolic pathway involving phase I metabolism due to cytochrome P450 (Herrera, et al., 2005). Because this comparative study also included an assessment of human microsomal activity (pooled from Caucasian, Hispanic and African American donors from 15 to 66 years of age), it was possible to conclude that metabolism in dogs and humans is similar.

Horses

In vitro metabolism of monensin also has been evaluated in microsomal incubates from a horse. Compared to the values obtained for humans and dogs, metabolic stability was highest in the horse and intrinsic clearance was lowest (Herrera, et al., 2005). This effect was exacerbated at high concentrations and reflects the toxicity seen in horses. The catalytic efficiency (chickens >> cattle >> rat/pig > horse) was found to correlate inversely with the interspecies differences in the susceptibility to toxic effects (Nebbia, et al., 2001).

Metabolism in Food Producing Animals

Cattle

Monensin is converted to a large number of metabolites in steers, with the most abundant (M-6) representing approximately 6% of the liver [^{14}C] residue (Donoho, et al,, 1978). A subsequent study in dairy cows reported a similar pattern of metabolites with the most abundant (again M-6) representing 24% of the liver total radioactivity (Kennington, et al., 1995). Six faecal metabolites (M-1 to M-6) were isolated and tentatively identified based on their mass spectral comparison to monensin (Donoho, et al., 1978). In faeces, the predominant residue is monensin (50%), followed by M-6 (4%) and M-2 (2%), respectively. O-demethylation is a major metabolic pathway (Donoho, et al., 1978; Kennington, et al., 1995).

Three steers were fed 300 mg unlabelled monensin per day for at least 15 days. The steers were then given single doses of approximately 300 mg [^{14}C] monensin (specific activities 0.027 - 0.030µCi/mg). Animals received unlabelled monensin for the final 14 days. Radioactivity in faeces remained above background for seven to 11 days. The proportion of the dose recovered was 88.6% - 102.3%. Urine contained no radioactivity above the pre-dose level (Herberg, 1973c; Herberg, 1974a).

Pigs

Monensin and metabolites M1, M2, and M8 were identified in liver of pigs (Giera, et al., 1984a).

Two balance-excretion experiments were conducted in pigs using barrows that had been conditioned to diets containing 50 mg monensin/kg feed then given single doses of [^{14}C] monensin. Doses used were 10.4 mg (specific activity 0.576 µCi/mg) and 5.23 mg (specific activity 0.608 µCi/mg). Recoveries were 78.1% and 54.9% of the doses over ten and 13 days, respectively (Donoho and Herberg, 1977; Herberg and Donoho, 1977a). In the high dose study, 75.0% of the dose was recovered in faeces and 3.1% in urine. In the low dose study, recoveries were 53.9% in faeces and 1.0% in urine. In both studies, excretion was rapid, with approximately 92% of the total in faeces recovered within the first 3 to 3½ days.

Sheep/Goats

In lambs dosed orally with [^{14}C] monensin, liver residues of monensin (6-9% of total radioactivity), M-6 (4-6% of the total), M-1 (5-10% of total) and M-2 (5-9% of total) were identified by TLC radioautography and comparison with standards (Giera, et al., 1984b). In faeces, the major residue was determined to be parent monensin (approximately 75% of the total radioactivity). Small amounts of M-1 (4% of the total) and M-2 (5% of the total) also were identified in sheep faeces (Giera, et al., 1984b).

A wether lamb was given a single dose of 50 mg unlabelled monensin for two weeks. The lamb then received a single 50 mg dose of radiolabelled monensin (0.027µCi/mg). For the final two weeks, the lamb again received unlabelled monensin. Radioactivity in faeces remained above background for nine days. The total amount of radioactivity recovered was 102.0% of the dose. Urine contained no radioactivity above the pre-dose level (Elanco, 1998).

Monensin also was detected in the liver of goats (Handy and Rea, 1984).

Chickens/Turkeys

In chickens, radiochromatograms show extensive metabolism of monensin. The identification and quantification of all metabolites was not possible. Liver (at practical zero withdrawal), bile (at zero and one day withdrawal) and excreta (on the sixth day of dosing) were collected for analysis. Monensin metabolites in liver, bile and excreta included O-demethylation and oxidation (hydroxylation) products. Parent monensin was present in liver and excreta but not bile. Monensin metabolites M-1, M-2, M-6, M-7, and M-9 were identified in liver, bile, and excreta (Grundy, et al., 1998). Metabolites M-7 and M-9 had not been identified in earlier TLC/autoradiography determinations (Donoho, et al., 1980a; Donoho, et al., 1982b).

Figure 2:

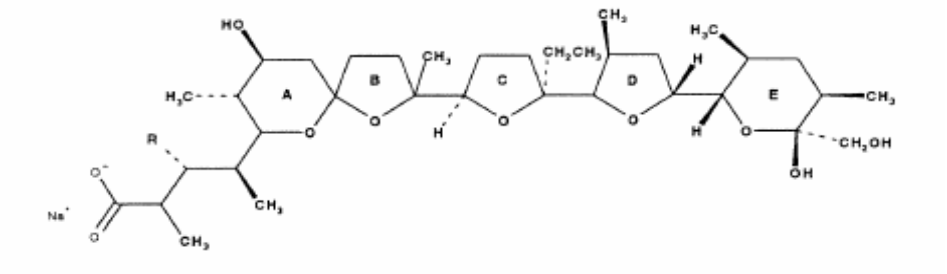

Table 2: Summary of monensin metabolites isolated from animal tissues and excreta. (Structural positions refer to Figure 2)

Metabolite	Molecular weight	Properties	R	Other	Source
M-1	678	O-demethylated monensin (hydroxylated)	OH	-	C, R, T, Ch, Sh, S
M-2	694	O-demethylated monensin (hydroxylated in two positions)	OH	Additional OH on ring E	C,R, T, Ch, Sh, S
M-3	694	M-2 epimer (hydroxylated in two positions)	OH	Additional OH on ring E	R,T, Ch
M-4	694	O-demethylated monensin (hydroxylated in two positions)	OH	Additional OH on ring D	C, R, T
M-5	708	Monensin sodium + oxygen (hydroxylated)	OCH_3	Additional OH on ring D	C
M-6	610	O-demethylated monensin with oxidation of the OCH_3 group to a ketone	keto	Carboxyl group absent	C, R, T, Ch, Sh
M-7	694	Isomeric with M-2, M-3, M-4 but with the oxygen at a different location (hydroxylated in two positions)	OH	Additional OH on ring B, C, or D	R, Ch
M-8	-	O-demethylated monensin (hydroxylated in two positions)	OH	Additional OH on ring B, C, or D	S
M-9	-	O-demethylated monensin with oxidation of the OCH_3 group to a ketone + additional OH	keto	No carboxyl group; additional OH on ring E	Ch

C = Metabolite identified in cattle liver, bile and/or excreta
R = Metabolite identified in rat tissues and/or excreta
T = Metabolite identified in turkey tissues and/or excreta
Ch = Metabolite identified in chicken tissues and/or excreta
S = Metabolite identified in pig tissues and/or excreta
Sh = Metabolite identified in sheep tissue and/or excreta

TISSUE RESIDUE DEPLETION STUDIES

Radiolabelled Residue Depletion Studies

Cattle

A number of early studies in steers orally dosed with 300 mg [^{14}C] monensin/kg body weight indicated that essentially all of the radioactivity could be recovered in the faeces within 11 days after dosing, but no radioactivity above the predose period could be recovered in the urine (Herberg, 1973c; Herberg,1973d; Herberg, 1974a; Herberg, 1974b; Herberg, 1974c). A subsequent study with similar doses of [^{14}C] monensin for a five-day period indicated that the liver contained the greatest concentration of radioactivity 12 hours after the last dose (Herberg, et al., 1978).

The distribution of radiolabelled monensin (Day, et al., 1973) residues in cattle tissue obtained at zero withdrawal is summarized in Table 3 (Herberg, 1975b; Herberg, et al., 1978,

Donoho, et al., 1978; Donoho, 1979). Animals were slaughtered after a practical zero withdrawal, six to twelve hours after the last dose. Cattle were dosed orally by gavage with a gelatine capsule, twice daily for two to five days. The daily dose was 0.71 to 0.83 mg [^{14}C] monensin/kg body weight, corresponding to 300 to 330 mg [^{14}C] monensin per day. An equivalent dose of monensin in feed would be 33 mg monensin/kg feed to 44 mg monensin/kg feed. One steer was preconditioned for twelve days with an equal dose of unlabelled monensin provided in feed. The remaining cattle were withdrawn from monensin-treated feed three days prior to receiving [^{14}C] monensin.

Table 3: Summary of total radioactive residues (mg equivalents/kg) at zero withdrawal in tissues of cattle dosed orally with [^{14}C] monensin.

Dose Equivalent	Dosing Interval	Animal	Total Radioactive Residue (mg monensin equivalents/kg)				
			Liver	Kidney	Heart	Muscle	Fat
44 mg monensin/kg feed	2 days	Steer	0.59	0.03	0.01	0.01	0.05 (back)
							0.04 (kidney)
		Steer 518	0.43	0.01	0.01	0.01	NDR[1]
33 mg monensin/kg feed	5 days	Steer 558	0.36	0.01	NDR	0.01	0.02
		Heifer 074	0.21	0.01	0.01	0.02	NDR

[1] No detectable residue (not statistically different from control tissues).

A radiolabelled residue study was conducted in five lactating dairy cows. Animals received 0.9 mg [^{14}C] monensin/kg body weight in a gelatine capsule twice daily *via* rumen cannulae for 9½ days (Kennington, et al., 1995). The total daily dose ranged from 918 to 1125 mg [^{14}C] monensin/day corresponding to approximately 36 mg monensin/kg feed (1.5 times the labelled dose). As in other studies, liver was the edible tissue with the highest mean residue at practical zero withdrawal times (Table 4). Mean muscle residues were below the assay limit of detection (28.4 cpm in muscle). Total residues in milk are reported below.

Table 4: Mean radioactivity (mg monensin equivalents/kg) at zero withdrawal in tissues of dairy cows administered 918 to 1125 mg [^{14}C] monensin/day.

Tissue	Total Radioactive Residue (mg monensin equivalents/kg)
Liver	1.28
Kidney	0.07
Muscle	NDR
Fat	0.02

Pigs

In an early study, [^{14}C] monensin, at a nominal concentration of 55 mg monensin/kg feed, was fed to one barrow and three gilts for five days (Herberg and Donoho, 1977b). One barrow and one gilt were sacrificed after five days of feeding as a zero-time withdrawal pair. The remaining two gilts were sacrificed after 24 and 48 hours withdrawal. At all withdrawal times, liver had the greatest radioactivity concentration. Zero-withdrawal time liver concentrations were 1.67 and 1.20 mg monensin equivalents/kg for barrow and gilt, respectively. The net radioactivity concentrations in muscle tissue were < 0.05 mg monensin/kg at all times. All tissues other than intestine and pancreas at all withdrawal times contained some residue of radioactivity (<0.09 mg monensin equivalents/kg).

In a preliminary study, one male and one female pig were dosed orally for two and one-half days with [^{14}C] monensin at an equivalent to 50 mg monensin/kg in feed (Herberg and Donoho, 1978). Four hours after the final dose, the pigs were sacrificed and edible tissues were assayed for residual activity. Liver contained the highest net residue, 1.02 mg monensin equivalents/kg in the male and 1.44 mg monensin equivalents/kg in the female. Residues in the other tissues were less than 0.09 mg monensin equivalents/kg.

Three grower pigs of each sex were fed [^{14}C] monensin-fortified ration (110 mg monensin/kg feed) for five consecutive days (Giera, et al., 1984a). One male and one female were slaughtered at six hours, three days and five days withdrawal. Liver, kidney, fat, and muscle were assayed for total radioactivity (limit 0.05 mg/kg). Bioautography was used for detection of monensin in those tissues. The bio-autographic method (Rea, 1976) has a limit of detection of 0.025 mg/kg for liver and muscle and 0.05 mg/kg for fat and kidney. Selected samples of liver and faeces were characterized chromatographically (LOD = 0.005 mg/kg).

The radioactive residues found in pig tissues are summarized in Table 5. Liver from the male and female zero-time withdrawal animals contained approximately 2.26 mg monensin equivalents/kg total residue. Residues decreased to approximately 0.44 mg monensin equivalents/kg by 5 days withdrawal. Kidney contained approximately 0.17 mg monensin equivalents/kg total residue at zero withdrawal. Residues decreased to approximately 0.05 mg monensin equivalents/kg after 5 days. The radioactive residues in fat and muscle were approximately 0.044 and 0.037 mg monensin equivalents/kg, respectively, at zero withdrawal. After five days of withdrawal, fat and muscle ^{14}C residues were approximately 0.05 and 0.02 mg monensin equivalents/kg, respectively. Monensin was not detected in the tissues of any of the treated animals by a microbiological assay (sensitivity 0.025-0.050 mg/kg) or HPLC assay (sensitivity 0.005 mg/kg).

Table 5: Net mg ^{14}C-monensin/kg equivalents in pig tissues following oral administration of radiolabelled monensin in feed (110 mg/kg).

	0-Day (6hr) Withdrawal		3-Day Withdrawal		5-Day Withdrawal	
	F 126	M 127	F 122	M 125	F 121	M 120
Liver	2.08	2.45	0.88	1.03	0.35	0.53
Kidney	0.16	0.18	0.08	0.09	0.04	0.06
Fat	0.04	0.05	0.05	0.05	0.03	0.06
Muscle	0.04	0.04	0.02	0.02	0.02	0.02

Sheep

Lambs were fed [^{14}C] monensin equivalent to a feeding level of 15g per ton (16.5 mg/kg) of complete ration (Giera, et al., 1984). Groups of lambs (two wethers and one ewe) were dosed for 3, 5, or 7 days and killed at zero withdrawal (12 hours) after the last dose. Edible tissues were assayed for total radioactivity (assay reliability = 0.1 mg/kg or lower). Liver samples were assayed for parent monensin, and selected samples of liver and faeces were characterized chromatographically. After dosing for 3, 5, or 7 days, liver contained mean residues of 0.36, 0.32, and 0.20 mg/kg radioactivity equivalents, respectively (Table 6). Parent monensin concentrations in liver were determined using bioautography (Kline, et al., 1975) and were less than 0.05 mg/kg. There was no accumulation of residues for either total radioactivity or parent monensin with longer dosing intervals. Residues in kidney and fat were all less than 0.03 mg monensin equivalents/kg and muscle all less than 0.01 mg monensin equivalents/kg.

Table 6: Summary of ^{14}C (mg monensin equivalents/kg) in sheep tissues following oral administration of radiolabelled monensin at 15 g/ton in feed.

	3 Day Dosing Period			5 Day Dosing Period			7 Day Dosing Period		
	M 578	F 579	M 583	M 580	M 581	F 582	M 577	M 584	F 585
Liver[1]	0.50	0.18	0.39	0.40	0.29	0.285	0.32	0.19	0.11
Kidney	0.01	0.004	0.01	0.02	0.01	-0.01	0.01	-0.01	0.01
Fat	0.01	0.01	0.01	0.01	0.02	0.01	0.03	0.01	0.01
Muscle	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

Chickens/Turkeys

The distribution of radiolabelled residues at zero withdrawal has been extensively evaluated in chickens and turkeys.

In several studies, chickens were treated with [^{14}C] monensin in feed at 110-125 mg/kg, *ad libitum*, for four to six days. The first study (Table 7) established that steady-state equilibrium occurred within four days in chickens receiving 120 mg [^{14}C] monensin/kg in feed (Donoho, et al., 1980a). In all [^{14}C] monensin residue studies, liver tissue had the highest amount of total residues at a practical or true zero withdrawal. Muscle had the least residues at zero withdrawal. This was consistent with the distribution pattern seen in rats (Howard and Lobb, 1981).

Table 7: Mean radioactivity (mg monensin equivalents/kg) at zero withdrawal in tissues of chickens fed 110 to 125 mg [^{14}C] monensin/kg feed.

Study No.	Dose (in feed)	Dosing Interval	Total Radioactive Residue (mg monensin equivalents/kg)				
			Liver	Kidney	Muscle	Skin/Fat	Fat
ABC-0043[1]	120 mg/kg	4 days	0.56	0.12	0.01	0.09	0.14
		6 days	0.43	0.12	0.01	0.07	0.07
ABC-0080[2]	120 mg/kg	5 days	0.83	0.22	0.05	0.29	0.23
ABC-0092[3]	110 mg/kg	5 days	0.53	0.18	0.02	0.12	0.07
T1F759701[4]	125 mg/kg	6 days	0.94	0.20	0.06	0.29	0.48

[1]Donoho, et al., 1980a; [2]Donoho, et al., 1980b; [3]Donoho, et al., 1980c; [4]Grundy, et al., 1998

The depletion of [^{14}C] monensin is summarized in two studies (Donoho, et al., 1980c; Grundy, et al., 1998). In the first study (Table 8), fat and skin/fat had slowly depleting residues. The relatively high and persistent radioactivity in abdominal fat was due to incorporation of radioactivity into endogenous fatty acids (Grundy, et al., 1998). Total residue in liver was approximately 2-15 times greater than residues in kidney, muscle, abdominal fat, and skin/fat at zero withdrawal. After three days, liver residues depleted to less than the fat and skin residues.

Fat was also a slowly depleting tissue in the second study (Table 9, Donoho, et al., 1980c). In the liver, the percentage of bound (unextracted) radioactivity increased from 38 to 69% during the 5-day withdrawal even as the total liver radioactivity decreased. Over the 5-day study, the absolute amount of bound residue in liver decreased from approximately 0.20 mg/kg to 0.07 mg/kg.

Table 8: Summary of total radioactive residues (mg monensin equivalents/kg) in edible tissues, fat, and bile of chickens after *ad libitum* access to 125 mg [^{14}C] monensin/kg feed.

Treatment Group	Withdrawal Time (days)	Total Radioactive Residue (mg monensin equivalents/kg)					
		Liver	Kidney	Muscle	Skin/Fat	Abdominal Fat	Bile
01	0	0.94	0.20	0.06	0.29	0.48	87
02	1	0.49	0.14	0.05	0.17	0.31	26
03	3	0.27	0.09	0.05	0.23	0.47	1.3
04	5	0.14	0.05	0.04	0.17	0.26	0.4

Table 9: Summary of total radioactive residues (mg monensin equivalents/kg) in tissues of chickens fed 100 mg [^{14}C] monensin/kg in feed.

Withdrawal Time (days)	Total Radioactive Residue (mg monensin equivalents/kg)				
	Liver	Kidney	Muscle	Skin	Fat
0	0.53	0.18	0.02	0.12	0.07
1	0.27	0.08	0.01	0.05	0.07
2	0.22	0.06	0.01	0.06	0.07
3	0.15	0.05	0.01	0.04	0.05
5	0.11	0.04	0.01	0.03	0.05

In turkeys fed 110 mg [^{14}C] monensin/kg in feed for five days, radiolabelled residues were evaluated in the edible tissues of birds killed 6 hours (practical zero withdrawal) after the removal of medicated feed (Donoho, et al., 1982a). As in the chicken studies, turkey liver and muscle had the most and least amount of radioactive residue, respectively (Table 10).

Table 10: Total radioactive residue in turkey tissues at zero withdrawal following treatment with 110 mg [^{14}C] monensin/kg feed for five days.

Tissue	Total Radioactive Residue (mg monensin equivalents/kg)
Liver	0.91
Kidney	0.16
Muscle	<0.03
Skin/Fat	0.10
Fat	0.14

Cows' Milk

Radiolabelled residues in milk were determined in a study conducted in lactating dairy cows (Kennington, et al., 1995). Cows were treated intra-ruminally *via* gelatine capsule for 9 days. Within approximately five days, radiolabelled milk residues had reached a steady state and averaged 0.045 mg/kg during the final three days of dosing. No residues of monensin were detected in the milk of any treated animals (LOQ = 0.005 mg/kg in milk). The radioactivity was distributed approximately 30% in the cream, 50% in the whey, and 20% in the casein. Fractionation of the milk and the use of a sensitive LC/ESP-MS/LSC analytical technique allowed for the detection of parent monensin in milk at low concentrations (<0.001 mg/kg). As in chickens (Grundy, et al., 1998), it was determined that a significant proportion (26.5%) of the radioactivity in milk from an animal treated with [^{14}C] monensin was due to the incorporation of the radioactivity into endogenous fatty acids (myristic, oleic, palmitic and stearic acid), rather than resulting from monensin-related residues (Grundy and Bewley, 2003).

Residue Depletion Studies with Unlabelled Drug

Residues in Tissues

Cattle

Monensin was fed to cattle at levels of 100 and 500 mg/animal/day for 148 days and at 750 mg/animal/day for 106 days (Kline, 1973). Animals were slaughtered at 0, 48, 120 and 240 hours withdrawal for the 100 and 500 mg/animal/day treatments and at 0 and 48 hours withdrawal for the 750 mg/animal/day treatment. No monensin residues (assay sensitivity 0.05 mg/kg) were detected in animals receiving 100 or 500 mg monensin/day at any withdrawal period. One kidney sample from a zero withdrawal animal in the 750 mg/animal/day treatment group contained a detectable residue. At 48 hours withdrawal, tissues in the 750 mg/animal/day treatment group were free of residual monensin.

Lactating dairy cows were treated intra-ruminally with two controlled release capsules (32 g monensin in a hexaglycerol distearate matrix into a plastic tube) and fed a medicated ration containing 24 mg monensin/kg feed for 10 days and then fed 36 mg monensin/kg for 21 days (Bagg and Dick, 1999). After measurement of the monensin release rate from the controlled release capsules, the resulting daily dose ranged from 1537 to 1804 mg monensin per cow. At zero withdrawal, animals were slaughtered and liver and kidney samples were analyzed using a validated HPLC method with post-column derivatization. There were no detectable monensin residues in kidney tissue (<0.025 mg/kg). Monensin residues were detected in 4 of 6 liver samples (detected residues ranged from 0.05 mg/kg to 0.09 mg/kg).

To determine monensin residues in the tissues of lactating dairy animals, Holstein cows were fed medicated rations containing 0, 24, or 36 mg monensin/kg feed or with 1.8 mg monensin/kg body weight *via* gelatine capsule through a rumen fistula (Dick, et al., 1994). At the completion of feeding periods, liver was analyzed using a validated HPLC method with post-column derivatization. No residues were found above the method LOQ of 0.025 mg/kg.

The depletion of monensin was determined in the edible tissues (liver, muscle, kidney and fat) of 12 lactating dairy cows after dosing with monensin at 0.9 mg/kg body weight for seven consecutive days (Bassissi and Larvor, 2007). Gelatine capsules containing equal doses were administered at approximately 12-hour intervals. Tissues were collected at 6, 18 and 30 hours after the final dosing. Monensin residues (Table 11) were determined using a validated HPLC-MS/MS method with a LOQ of 1 µg/kg.

Table 11: Residues of monensin in the tissues of dairy cows treated *via* gelatine capsule at 0.9 mg/kg body weight in two equal doses for 7 days.

		Concentration (µg/kg)			
Animal No.	Time after last dosing (hours)	Muscle	Fat	Liver	Kidney
25	6	BLQ	5.2	9.6	1.03
32		BLQ	3.2	9.4	BLQ
39		ND	BLQ	6.4	BLQ
43		BLQ	1.1	10.8	BLQ
16	18	ND	BLQ	4.8	BLQ
71		ND	1.4	5.2	BLQ
15		BLQ	BLQ	5.4	BLQ
72		ND	BLQ	6.7	BLQ
3	30	ND	BLQ	2.2	ND
11		ND	BLQ	2.3	ND
75		ND	BLQ	5.4	ND
77		ND	BLQ	2.4	ND

ND = below the limit of detection. BLQ = below the lower limit of quantification (LOQ = 1 µg/kg). Bassissi and Larvor, 2007.

Pigs

Growing-finishing pigs received a medicated feed containing 100 mg monensin/kg feed for 98 days (Handy and Rea, 1976). Randomly selected animals were continued on medicated feed for two days or transferred to a non-medicated diet for two days. Animals were then slaughtered (effectively zero and 48 hours withdrawal). Edible tissues were analyzed for monensin by bio-autography (Rea, 1976). No monensin residues were detected in any of the tissues assayed. The assay has a sensitivity of <0.05 mg/kg for muscle and <0.025 mg/kg for liver, kidney, and fat.

Sheep/Goats

Wether lambs were treated *ad libitum* for 118 days with a medicated ration containing 0, 10, 20, or 30 g monensin/ton (11, 22 or 33 mg/kg) (Kline et al., 1975). Medicated feed was replaced with non-medicated feed and animals were withdrawn for 0, 24, or 48 hours. Samples of muscle, fat, liver, and kidney were collected and analyzed using a bioautography procedure (Kline and Wicker, 1975). No detectable monensin residues were found in samples of muscle, fat, and kidney from animals at any of the withdrawal times for any of the doses. Monensin was detected in liver samples collected at zero withdrawal. Activity below the test sensitivity of 0.05 mg/kg also was found in several liver samples at 24 h withdrawal. No residues were detected at 48 hours withdrawal.

Male Angora goats were fed rations containing 0, 20, or 30 g monensin/ton (11, 22 or 33 mg/kg) of feed for 56 days (Handy and Rea, 1984). Animals were withdrawn from medicated feed for zero or five days. At slaughter, liver samples were collected and analyzed by a bioautography procedure (Kline and Wicker, 1975). Monensin was detected in half of the 33 mg monensin/kg treatment liver samples and about 20% of the 22 mg monensin/kg treatment samples collected at zero withdrawal. One sample contained 0.04 mg/kg monensin (LOQ = 0.04 mg/kg). No monensin was detected in any of the 5-day samples.

Chickens

In one study (Callender, et al., 1980), male Hubbard chickens were reared for 45 days on feed containing 110 g monensin/ton (120 mg/kg). The birds were then placed on feed containing 15, 45, or 110 g monensin/ton (16.5, 50, or 120 mg/kg) for five days. At slaughter, abdominal fat, liver, and breast muscle tissues were assayed for monensin residues. No residues of monensin were found in liver and muscle tissue from any of the birds (LOQ = 0.04 mg/kg). Concentrations of monensin <0.04 mg monensin/kg were detected in fat tissue from birds in the 120 mg monensin/kg treatment group. No residues were found in the fat tissues from birds treated with feed containing 16.5 or 50 mg monensin/kg.

In an earlier study, chickens were fed monensin (120 mg monensin/kg) alone or in combination with other feed additives (Callender, 1978). Tissues were analyzed with a bioautography method with a sensitivity of approximately 0.05 mg/kg (Donoho and Kline, 1967). Residues are reported as samples positive/total samples. More than 2000 samples were analyzed. Although a few samples were positive at 0 and 24 hours withdrawal, samples from chickens withdrawn for 48, 72, and 96 hours were all negative. The study concludes that there was little potential for residues to exceed the 0.05 mg/kg concentration when chickens were fed monensin and withheld for 24 hours or more.

In studies (Pankhurst, 1981) where monensin was fed to boiler chickens at the highest recommended level (120 mg monensin/kg feed), the concentrations of monensin were determined with a bioautography method (Donoho and Kline, 1967). Samples were reported as positive/negative for monensin. While a limited number of fat (18/22), muscle and liver (2/12), and kidney (1/16) contained detectable concentrations of monensin at zero withdrawal, all samples at 24 or 48 hours were negative.

In another study (Okada, et al., 1980), chickens received medicated feed containing 80, 100, or 120 mg monensin/kg feed for 9 weeks. Thin-layer bioautography was used to determine the concentrations of monensin in edible tissues (Donoho and Kline, 1967). The minimum detection limits for the method used were 0.01 mg/kg in fat and 0.0125 mg/kg in liver, kidney, and muscle. Concentrations of monensin residues at zero withdrawal were 0.06 to 0.11 mg/kg in fat, undetectable to 0.04 mg/kg in muscle, undetectable to 0.04 mg/kg in liver and undetectable to 0.01 mg/kg in kidney. No detectable residues of monensin were found in fat at 48 hours or longer withdrawal times, or in liver, muscle, and kidney at 24 hours or more after withdrawal. Tissue residue concentrations did not increase proportionally when feed concentrations were increased to 300 and 600 mg monensin/kg feed.

The depletion of monensin from edible tissues was evaluated in broiler chickens (Atef, et al., 1993) following administration by gavage as a single dose of 40 mg monensin/kg body weight. Monensin residues were detected in all tested tissues (liver, kidney, fat, skin, thigh and breast muscle, plus heart) collected 2, 4, 6, and 8 hours after administration. The highest concentrations of monensin residues were found in liver. Twenty-four hours after administration, monensin residues were detected in liver, kidney and fat. Monensin residues were detected only in liver 48 hours after administration.

In a recent study, 30 chickens were treated with monensin sodium in the diet (nominal level of 125 mg monensin/kg in feed) for 42 consecutive days (Walker and McLean, 2007). The birds were sacrificed at specified time points after removal of the medicated diet. Samples of liver, kidney, muscle, and skin with fat were collected for analysis using a validated HPLC method with post-column derivatization and UV detection at 520 nm. The assay LOQ was 0.025 mg/kg. At zero withdrawal, the highest concentration of monensin (factor A) was found in skin with fat (0.02 mg/kg) followed by liver (0.02 mg/kg) and kidney (0.01 mg/kg). No monensin residues were detected in muscle samples. There were no detectable residues in any

tissues collected at 12 or 48 hours withdrawal. Samples collected at 48 and 72 hours withdrawal were not analyzed.

In another recent study, a sensitive LC-MS/MS method was developed and validated for the quantitation of monensin in the edible tissues of chicken (Chéneau, et al., 2007). In a subsequent depletion study, 68 chickens were treated orally with monensin (121 mg monensin/kg feed) for 33 days. The residues declined rapidly and were only observed in fat at the 18 hour sampling.

Unpublished data also are available (Sanders, 2008a; Henri, et al., 2008b). In this study, samples were collected at close intervals. The data are presented in Table 12.

Table 12: Tissue residues (µg/kg) in chickens after feeding monensin in the diet at the rate of 125 mg/kg (mean ± SD).

Tissue	0 h	2 h	4 h	6 h	8 h
Liver	17.0 ± 6.4	4.9 ± 3.9	3.8 ± 1.8	1.5 ± 0.3	<LOQ
Fat	49.1 ± 20.5	29.2 ± 7.0	28.8 ± 10.2	10.5 ± 7.8	5.0 ± 2.1
Muscle	5.8 ± 2.0	6.35	<LOQ	3.4 ± 0.3	<LOQ
	10 h	12h	23 h	35 h	71 h
Liver	<LOQ	<LOQ	NA	NA	NA
Fat	9.3 ± 2.5	7.1 ± 5.4	<LOQ	<LOQ	<LOQ
Muscle	<LOQ	<LOQ	<LOQ	ND	<LOQ

LOQ: 1 µg/kg for liver, 2.5 µg/kg for fat and muscle. NA=not analysed. ND = not detected.

Turkeys

In a residue depletion study, turkeys received medicated feed containing monensin at 120 mg monensin/kg for approximately 17 weeks. Birds were withdrawn from medicated feed for 0, 24, 48, 72 and 96 hours. Edible tissues were collected at slaughter and analyzed for monensin using a bioautography method (Donoho, 1972). Detectable residues were found in all tissues at zero withdrawal. No residues of monensin were detected in fat and kidney samples beyond 24 hours withdrawal. Muscle and skin were free of residual monensin 48 hours post-treatment. Liver samples were negative at 72 hours (Donoho, 1972). Additional residue data for turkeys are provided in an unpublished study (Sanders, 2008b), Table 13.

Table 13: Tissue concentrations of monensin (µg/kg) in turkeys after feeding at the rate of 100 mg/kg monensin (mean ± SD).

Tissue	0 h	2 h	4 h	6 h
Liver	3.5 ± 1.2	1.7 ± 0.1	1.8 ± 1.0	1.8 ± 1.0
Fat	40.5 ± 7.4	33.8 ± 19.2	35.6 ± 14.1	42.4 ± 37.1
Muscle	4.5 ± 1.4	4.1 ± 0.0	2.5 ± 0.0	4.3 ± 1.6
	8 h	10 h	12 h	24 h
Liver	ND	ND	ND	ND
Fat	9.8 ± 4.3	6.1 ±3.3	4.2 ± 0.2	3.4 ± 0.6
Muscle	ND	ND	ND	ND

NA=not analyzed. ND=not detected.

Quail

Two groups of quail were reared for eight weeks. One group received non-medicated feed while the treatment group received feed containing 80 mg monensin/kg feed continuously throughout the growth period (Handy and Rea, 1985). At the end of the feeding period, the

birds were sacrificed with no withdrawal period. Liver tissues were pooled to provide 15 g samples from each group. Monensin residues were determined using a thin-layer bio-autography method with a LOQ of 0.04 mg monensin activity/kg in chicken and bovine tissues (Kline and Wicker, 1975). No monensin was detected in any of the liver samples from monensin-treated birds.

Residues in Milk and Eggs

Cows' Milk

In a milk residue depletion study, lactating dairy cows were treated with two monensin controlled release capsules and fed a medicated feed containing 36 mg monensin (Bagg and Dick, 1999), resulting in an average daily dose of 1804 mg monensin per cow. There were no detectable residues of monensin in milk (<0.005 mg/kg) while on treatment.

In another study, Holstein cows, approximately 80 to 120 days in milk with four functional quarters, were treated with a total mixed ration containing 0, 24, or 36 mg monensin/kg feed or administered a gelatine capsule (through a rumen fistula) containing 1.8 mg monensin/kg body weight. At the completion of feeding periods, the milk was assayed for monensin using a validated HPLC method with post-column derivatization (Dick, et al., 1994). No milk samples contained residues of monensin at or above the LOQ of the method (0.005 mg/kg).

Residue depletion in the milk of 12 lactating dairy cows was determined after dosing with monensin at 0.9 mg/kg body weight for seven consecutive days (Bassissi and Larvor, 2007). Gelatine capsules containing equal doses were administered at approximately 12-hour intervals. Milk samples were collected prior to the first treatment, on second milkings and on the third milkings after the last treatment. Monensin residues were determined using a validated HPLC-MS/MS method with a LOQ of 0.25 µg/kg. See Table 14.

Table 14: Concentrations of monensin in the milk of individual dairy cows treated *via* gelatine capsule at 0.9 mg/kg body weight in two equal doses for 7 days.

	Monensin residues (µg/kg)			
		Post-final-treatment		
Animal number	Pre-treatment	Milking 1	Milking 2	Milking 3
3	ND	0.54	BLQ	NSC
75	ND	0.38	BLQ	NSC
77	ND	0.32	BLQ	NSC
11	ND	0.39	BLQ	NSC
12	ND	0.41	BLQ	BLQ
19	ND	0.41	ND	ND
52	ND	0.48	0.32	BLQ
76	ND	BLQ	ND	ND

ND = below the limit of detection. BLQ = below the lower limit of quantification (LOQ = 0. 25 µg/kg). NSC = no specimen collected. (Bassissi and Larvor, 2007).

Effect on dairy starter cultures

Although no data on the effect of monensin on dairy starter cultures were included in the dossier, information is available in the EMEA summary report (EMEA, 2007). According to the summary report, monensin was tested against a panel of dairy starter cultures. *Lactobacillus acidophilus* La-5 (MIC equal to 1 µg/ml) and *Streptococcus thermophilus* TH-4 (MIC equal to 2 µg/ml) cultures were found to be the most sensitive to monensin. For all

other cultures, monensin MICs were 4 μg/ml or above. The summary notes that *Lactobacillus lactis* was not included in the test panel. The EMEA established the NOEL of monensin for commercial dairy starter cultures at 0.1 μg/ml (EMEA, 2007).

METHODS OF ANALYSIS FOR RESIDUES IN TISSUES

The earliest semi-quantitative method for the analysis of monensin in animal tissues and fluids was based on thin layer chromatography/bio-autography (Donoho and Kline, 1967; Donoho, 1984). It has a limit of detection of 0.025 mg/kg and a routine performance limit of 0.05 mg/kg. It has been refined to have a detection limit of 0.01 mg/kg (Okada, et al., 1980).

Residues of monensin in milk and tissues also are analyzed using an HPLC method with post-column derivatization with vanillin and detection at 520 nm (Elanco AM-AA-CR-R174-AA791 for bovine tissues and milk; Elanco AM-AA-CR-R152-AA-791 for poultry tissues). The limit of quantification is 0.025 mg/kg for tissues and 0.005 mg/kg for milk.

More recently, a method utilizing extraction with an organic solvent and clean-up on solid-phase extraction columns followed by liquid chromatography tandem mass spectrometry (LC-MS/MS) using C_{18} columns and electrospray detection methods (Dubois, *et al.*, 2004) has been developed. This multi-residue LC-MS/MS method, which uses dinitrocarbanilide-d8, diclazuril-bis and nigericin as internal standards, is suitable for the determination of monensin residues in whole eggs and residues in bovine, porcine, and avian tissues including muscle, liver and fat with a sensitivity of ≤0.001 mg/kg. Chéneau, et al. also have validated an HPLC/MS/MS method for analysis of chicken tissues and plasma using a narasin internal standard (Chéneau, et al., 2007), Tables 15 and 16.

Table 15: Results of the regression analysis of the data of the standard calibration graphs.

Matrix	Curve	Intercept	Slope	Quadratic term	Weight	R^2
Plasma	1	0	9.160E−05	–	1	–
	2	0	9.475E−05	–	1	–
	3	0	9.125E−05	–	1	–
			RSD: 2.1%			
Muscle	1	0	8.785E−05	-	1	-
	2	0	8.205E−05	-	1	-
	3	0	7.975E−05	-	1	-
			RSD: 5.0%			
Fat	1	0	3.058E−05	-	1	-
	2	0	2.955E−05	-	1	-
	3	0	2.893E−05	-	1	-
			RSD: 2.8%			
Liver	1	4.813E−04	1.168E−04	2.457E−09	$1/x^2$	0.9987
	2	6.231E−04	1.338E−04	2.073E−09	$1/x^2$	0.9997
	3	5.383E−04	1.246E−04	1.784E−09	$1/x^2$	0.9978
			RSD: 6.8%			

Most recently, a validated HPLC-MS/MS method with ESI tandem mass spectrometry was developed in accordance with Good Laboratory Practice and European Guidelines for the establishment of MRLs for residues of veterinary medicinal products in foodstuffs of animal origin (Cordroc'h, 2007). Using liquid phase extraction and a narasin internal standard, separation is achieved with a reversed phase column and gradient elution. The method is summarized in Table 17.

Table 16: Detailed results of validation for all matrices (plasma, muscle, fat, and liver).

Mean introduced concentration (g/L (plasma) or (g/kg)	Trueness		Precision		Accuracy	
	Absolute bias (g/L or g/kg)	Recovery (%)	Repeatability (RSD%)	Intermediate precision (RSD%)	β-Expectation tolerance limit (g/L or g/kg)	Risk (%)
Plasma						
2.5	-0.002	99.2	8.5	13.3	[1.85, 3.11]	18.3
5	-0.07	98.5	8.8	8.8	[4.24, 5.62]	2.1
10	-0.30	97.0	3.7	3.7	[9.12, 10.29]	<0.1
25	-0.40	98.4	3.4	3.4	[23.27, 25.93]	<0.1
100	1.91	101.9	4.5	4.5	[94.87, 109.00]	<0.1
Muscle						
0.5	-0.21	58.7	10.9	10.9	[0.16, 0.43]	94.1
2.5	0.05	102.2	5.2	5.7	[2.34, 2.77]	0.1
5	-0.59	88.2	4.6	4.6	[4.07, 4.75]	0.6
10	-0.99	90.1	3.2	6.5	[7.78, 10.24]	12.1
100	-1.72	98.3	2.8	7.7	[82.87, 113.70]	10.1
Fat						
2.5	0.11	104.3	3.3	4.0	[2.34, 2.88]	<0.1
5	0.35	107.0	6.5	8.1	[4.23, 6.47]	6.4
10	0.05	105.4	3.0	4.3	[9.24, 11.85]	0.2
100	0.07	100.7	4.8	4.8	[88.73, 112.60]	<0.1
200	-14.00	93.0	1.9	2.8	[168.90, 203.10]	<0.1
Liver						
1	-0.02	97.9	6.4	6.4	[0.82, 1.14]	<0.1
2.5	0.12	105.0	8.3	8.3	[2.12, 3.13]	<0.1
5	0.23	104.5	8.9	9.4	[4.05, 6.41]	0.1
10	-0.80	92.0	11.8	11.8	[6.32, 12.09]	1.2
100	0.69	100.7	3.5	3.5	[92.18, 109.20]	<0.1

Table 17: Summary of performance characteristics for the validated HPLC-MS/MS method (Cordroc'h, 2007).

	Kidney	Liver	Fat	Muscle	Milk
Interference and carry over	No interference				
Selectivity	Selective against tylosin, tilmicosin, tulathromycin, salinomycin, amoxicillin, ampicillin, cloxacillin, benzylpenicillin, cefoperazone and thiopental				
Linearity (µg/kg; ng/ml for milk)	1.0 to 250.0	1.0 to 250.0	1.0 to 250.0	1.0 to 50.0	0.25 to 50.0
Regression (weighting factor)	$1/x^2$	$1/x^2$	$1/x^2$	$1/x^2$	$1/x^2$
LOQ (µg/kg; ng/ml for milk)	1.00	1.00	1.00	1.00	0.25
LOD (µg/kg; ng/ml for milk)	0.22	0.24	0.15	0.12	0.06
Within-run precision (%)	3.0 to 6.5	2.5 to 4.2	2.9 to 4.8	0.7 to 5.0	5.8 to 8.2
Between-run precision (%)	4.2 to 9.3	2.5 to 5.2	3.3 to 6.3	3.0 to 5.5	8.7 to 19.3
Accuracy (%)	-3.0 to +6.3	-2.0 to +0.2	+1.6 to +8.0	+1.0 to +2.0	-0.7 to +5.1
Stability in extract during analysis (*ca.*5°C)	24 hours	48 hours	48 hours	48 hours	48 hours
Stability after freeze-thaw cycles	3 cycles				
Stability in extract during analysis (*ca.*20°C)	72 days	71 days	64 days	93 days	89 days
50-fold dilution test Precision (%) Accuracy (%)	 15.6/19.2* -17.9/-18.7*	 4.5 -11.1	 7.9 -11.5	 2.9 -12.3	 2.6 +1.2

*Not validated

APPRAISAL

Monensin has not been reviewed previously by the Committee. Monensin is a polyether ionophore produced by *Streptomyces cinnamonensis*. It exhibits both antibacterial and anticoccidial activities. Monensin is used for the control of coccidiosis in poultry, cattle, sheep, and goats. It is used to improve the efficiency of rumen fermentation, increase weight gain and to control ketosis. In dry and lactating dairy cows, it is used to increase milk production.

Monensin is metabolized extensively; the metabolic profiles are qualitatively similar across many tested species. The rat appears to be a suitable species for toxicity testing of monensin and its metabolites. Metabolism rates vary by species, with horses showing slow metabolism and high sensitivity to monensin. Animal species have been classified as relatively insensitive (mice and poultry), moderately sensitive (rats, rabbits, pigs, and ruminants) and extremely sensitive (horses). Monensin is eliminated rapidly, primarily in the faeces.

Radiolabelled studies were conducted in cattle (including lactating dairy cows), pigs, sheep, chickens, and turkeys. Radiolabelled total residues in muscle were uniformly low, in some studies less than the method LOQ. At zero withdrawal, residues were highest in liver in all species. In most species, at most doses, residues at early withdrawal times were highest in liver, followed by kidney and fat. In

chickens, at a dose of 125 mg monensin/kg feed, residues in fat exceeded those in kidney at all withdrawal times and, at withdrawal periods greater than one day, residues in fat also exceeded those in liver. In lactating dairy cows, radiolabelled residues in milk reached steady state (0.045 mg/kg) after five days dosing. There were no detectable residues of monensin (LOQ = 0.005 mg/kg). Using a sensitive LC/ECP-MS/LSC method, parent monensin was detected at low concentrations (<0.001 mg/kg). Much of the detected radioactivity in milk is attributed to incorporation of the radiolabel into endogenous fatty acids.

Monensin is an appropriate marker residue for monensin residues in tissues and milk. It represents approximately 5% of the total residues in tissues and 2.7% in milk.

Residue depletion studies using unlabelled monensin have been conducted in cattle, pigs, sheep, goats, chickens, turkeys, and quail.

In early studies in cattle, few if any detectable monensin residues were found. An early study in Holstein cows treated with monensin in feed or capsules, no detectable residues were found in liver tissues (LOQ = 0.025 mg/kg). In a more recent study, lactating cattle were treated with controlled release capsules and fed a medicated ration. Low but quantifiable residues were found in liver tissues. Kidney residues were less than the LOQ of <0.025 mg/kg. In the most recent study in lactating dairy cows, monensin was administered in gelatine capsules administered twice daily. Tissues were analyzed with an HPLC MS/MS method (LOQ = 0.001 mg/kg). Muscle residues were below the LOQ at all sampling times as were most of the kidney residues. Residues in fat were detectable at 6 hours withdrawal (3 of 4 samples) and 18 hours withdrawal (1 of 4 samples) but not at 30 hours withdrawal. Detectable residues were found in all liver samples at all withdrawal times.

No detectable residues were found in pigs treated with monensin. The studies are more than 30 years old and the method had limited sensitivity (LOQ range 0.025-0.050 mg/kg).

In sheep and goats, detectable residues of monensin were found in liver at zero withdrawal. Residues also were detected in sheep liver samples collected at 24 hours withdrawal. In goats, only one liver sample at zero withdrawal had quantifiable residues (LOQ = 0.040 mg/kg). There were no detectable residues at withdrawal times greater than 24 hours.

Several residue depletion studies were conducted in chickens. In the earliest studies, more than 2000 samples were analyzed. Only a few samples were positive at 0 and 24 hours withdrawal (sensitivity = 0.05 mg/kg). In a subsequent study, no residues were detected in muscle and liver samples. At the highest dose, 110 g monensin/ton, detectable residues were found in fat samples. When chickens were treated with medicated feed containing 120 mg monensin/kg feed, limited numbers of zero withdrawal samples contained detectable residues. All of the samples collected at 24 and 48 hours withdrawal were negative for monensin. In still another study, TLC bio-autography was used to assess monensin residues. While detectable residues were found in fat at zero withdrawal, residues were significantly lower in muscle, liver, and kidney. No detectable residues were found after 24 hours (liver, muscle and kidney) or 48 hours (fat) withdrawal. Following administration by gavage, monensin residues were detected in all tested samples collected at 2, 4, 6, and 8 hours withdrawal. Highest concentrations were found in liver. At 24 hours, monensin residues were detected in liver, kidney and fat. Only the liver contained detectable residues at the 48-hour withdrawal time. In a recent study, monensin residues were determined using a validated HPLC with post-column derivatization and UV detection. At zero withdrawal, highest residues were found in skin with fat followed by liver and kidney. There were no detectable residues in muscle. There were no detectable residues in any tissues collected at 12 or 48 hours withdrawal. In another recent study, an HPLC-MS/MS method was used to measure monensin residues in the edible tissues of chickens. Residues declined rapidly and were detectable only in fat at the 18-hour sampling time.

Depletion studies also were conducted in turkeys and quail. In turkeys, no residues of monensin were detected in fat and kidney samples beyond 24 hours withdrawal; muscle and skin were free of residual

monensin 48 hours post-treatment; liver samples were negative at 72 hours withdrawal. In quail, no monensin was detected in any of the liver samples from monensin-treated birds using a thin-layer bioautography method.

Three milk residue studies were conducted. In the oldest study, none of the milk samples contained residues of monensin at or above the LOQ of the method (0.005 mg/kg). In a more recent study, cows were treated via controlled release capsules and medicated feed. There were no detectable (LOQ = 0.005 mg/kg) residues in milk, even for milk samples collected while on treatment. In the most recent study, cows were treated by gelatine capsule and monensin residues were determined using an HPLC-MS/MS method (LOQ = 0.25 µg/kg). Only in the first milking were residues consistently above the LOQ.

MAXIMUM RESIDUE LIMITS

In recommending MRLs for monensin, the Committee considered the following factors:

- An ADI of 0–10 µg/kg bw was established by the Committee based on a chronic toxicological end-point. This ADI is equivalent to up to 600µg monensin for a 60-kg person.
- Monensin is the marker residue in both tissues and milk.
- Monensin is extensively metabolized; monensin represents, conservatively, 5% of total residues in tissues and 2.7% in milk.
- Liver contains the highest concentration of total residues at zero withdrawal in all species tested. In chickens treated at the maximum dose of 125 mg/kg in feed, total residues in abdominal fat exceed those in liver at 3 and 5 days of withdrawal. Liver can serve as the target tissue.
- While residue data in the studies submitted were determined using several methods, newer methods include a validated HPLC method with post-column derivatization and a validated HPLC-MS/MS method. Both of these newer methods are suitable for routine monitoring.
- The MRLs recommended for poultry tissues were based on residue data from the unlabelled residue depletion studies. For cattle, the residue concentrations were determined using the validated HPLC with post-column derivatization method. For chickens and turkeys, the residue concentrations were determined using the validated HPLC-MS/MS method.
- The MRL recommended for cows' milk was based on unlabelled residue depletion data determined using the validated HPLC-MS/MS method. The recommended milk MRL is 8 times the LOQ (0.25 µg/kg) for that method.
- Because monensin is not currently approved for use in pigs, no MRLs were recommended for monensin residues in pig tissues.

The Committee recommended permanent MRLs for monensin in poultry (chicken, turkey and quail) tissues of 10 µg/kg in liver, kidney and muscle, and 100 µg/kg in fat. The Committee recommended permanent MRLs for monensin in ruminant (cattle, sheep and goat) tissues of 10 µg/kg in kidney and muscle, 20 µg/kg in liver, and 100 µg/kg in fat, and and 2 µg/kg in milk. Residues in all species are determined as monensin.

It was not possible to do an intake estimate for monensin because of the small number of residue data points. Using the model diet and marker to total residue ratios of 5% for tissues and 2.7% for milk, the MRLs recommended above would result in an intake of 301 µg/person per day (poultry tissues plus milk) or 321 µg/person per day (ruminant tissues plus milk), which represent 50% and 54% of the upper bound of the ADI, respectively.

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NARASIN

First draft prepared by
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IDENTITY

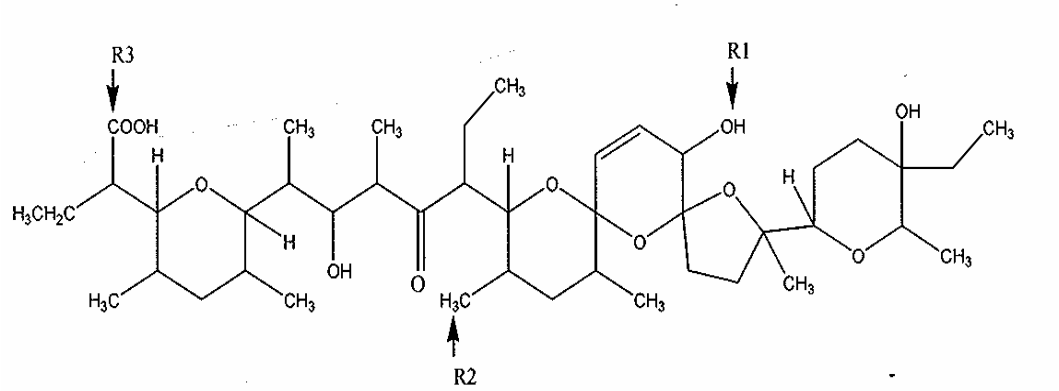
International Non-proprietary names (INN): Narasin

Synonyms: (4s)-4-methylsalinomycin, Narasin A, Monteban®, Naravin®

International Union of Pure and Applied Chemistry (IUPAC) Names: α-ethyl-6-[5-[2-(5 ethyltetrahydro-5-hydroxy-6-methyl-2H-pyran-2-yl)-15-hydroxy-2, 10, 12-trimethyl-1, 6, 8-trioxadispiro [4.1.5.3] pentadec-13-en-9-yl]-2-hydroxy-1, 3-dimethyl-4oxoheptyl] tetrahydro-3,5-dimethyl-2H-pyran-2-acetic acid.

Chemical Abstract Service (CAS) Number: 55134-13-9

Structural formula of main components:



Structural variants of Narasin	R1	R2	R3
A	OH	CH_3	COOH
B	=O	CH_3	COOH
D	OH	C_2H_5	COOH
I	OH	CH_3	$COOCH_3$

Molecular formula of Narasin A: $C_{43}H_{72}O_{11}$ (C 67.41%, H 9.49%, O 23.01%)

Molecular weight: 765.02

OTHER INFORMATION ON IDENTITY AND PROPERTIES

Pure active ingredient: Narasin A

Appearance: Crystal from acetone-water

Melting point: 98 - 100°C (crystal from acetone-water)
158 - 160ºC (crystalline narasin sodium salt)

Solubility: Soluble in alcohol, acetone, DMF, DMSO, benzene, chloroform, ethyl acetate. Insoluble in water.

RESIDUES IN FOOD AND THEIR EVALUATION

Conditions of use

Narasin belongs to the polyether monocarboxylic acid class of ionophores produced by *Streptomyces aureofaciens* strain NRRL 8092. Narasin is composed of 96% Narasin A, 1% Narasin B, 2% narasin D and 1% narasin I. The biological activity of narasin is based on its ability to form lipid soluble and dynamically reversible complexes with cations, preferably monovalent cations such as alkaline K^+, Na^+ and Rb^+: Narasin functions as a carrier of these ions, mediating an electrically neutral exchange-diffusion type of ion transport across the membranes. The resultant changes in transmembrane ion gradients and electrical potentials produce critical effects on cellular function and metabolism of coccidia. Narasin is effective against sporozoites and early and late asexual stages of coccidia in broilers caused by *Eimeria acervulina, E. brunetti, E. maxima, E. mivati, E. necatrix* and *E. tenella*. Narasin also is used for prevention of necrotic enteritis in broiler chicken.

The antimicrobial spectrum of activity of narasin is limited mainly to Gram-positive bacteria including *Enterococcus* spp*., Staphylococcus* spp., and *Clostridium perfringens*. Narasin is not used in human medicine and it is not classified as a critically important antibiotic for human use by expert meetings convened by WHO (WHO, 2007). It has, however been classified by OIE (OIE, 2007) as an important antibiotic for veterinary medicine for control of coccidiosis.

Dosage

Narasin has been approved for use in chickens for fattening at dose of 60-80 mg of active substance/kg of complete feed (54-72 gram per 2000 lb ton).

PHARMACOKINETICS AND METABOLISM

Because the principal effect of narasin is on the microflora of the intestinal tract (including coccidia); few conventional pharmacokinetic studies have been performed. Studies in both target and laboratory animals indicate that narasin is rapidly metabolised in liver and eliminated in faeces within a few days.

Pharmacokinetics in Laboratory Animals

Rats

A non-GLP compliant metabolism study was performed in rats in order to evaluate the absorption and excretion of narasin (Manthey, 1977a). A single oral dose of 2.3 mg of ^{14}C-labelled narasin with a specific activity of 0.596 µCi/mg was used. Rats were maintained in metabolism cages designed to separate the urine from the faeces. Food and water were provided *ad libitum.* Total radioactivity recovered in the urine and faeces was 75% of the administered dose at 52 hrs post-dosing. Only 1.1% of the total excreted radioactivity was found in the urine and the remainder was in the faeces (98.9%). In a study with three young mature rats surgically prepared for bile collection, approximately 15% of the dose was recovered in the bile samples indicating that a substantial portion of the ^{14}C narasin dose was absorbed and processed through the hepatic system.

Pharmacokinetics in Food Animals

Chickens

Three non-GLP compliant studies were evaluated.

In the first study (Peippo, et al., 2005), 30 males and 30 females broilers chickens (Ross 508-hybrid) were fed an un-medicated starter broiler ration from one-day -old until two weeks of age. For the duration of the study, chickens were fed a grower ration that contained 0, 3.5 or 70 mg narasin/kg of feed. Throughout the study, water and feed were supplied *ad libitum*. During the withdrawal period, chickens were again fed a non-medicated grower feed. At slaughter, samples of muscle were removed and blood was collected into heparin tubes. All the samples were stored at -20°C until analysed. Concentrations of narasin in the plasma and muscle of chickens were determined by time-resolved fluoroimmunoassay and results are shown in Table 1.

Table 1: Concentrations of narasin in plasma and muscle of broilers treated with 3.5 or 70 mg narasin/kg feed.

Feeding conditions	Bird number	Narasin concentration		
		Plasma (µg/L)	Leg muscle (µg/kg)	Breast muscle (µg/kg)
Feed containing 0 mg/kg of narasin	1	ND	ND	ND
	2	ND	ND	ND
	3	ND	ND	ND
	4	ND	ND	ND
Feed containing 3.5 mg narasin /kg; no withdrawal period	1	1.6	0.7	1.2
	2	1.8	0.6	0.7
	3	4.2	1.7	0.6
	4	3.4	1.6	1.3
Feed containing 70 mg narasin /kg; no withdrawal period	1	39.8	2.4	2.1
	2	59.3	4.2	2.3
	3	70.2	6.2	4.5
Feed containing 70 mg narasin /kg; 3 day withdrawal period	1	ND	ND	ND
	2	ND	ND	ND
	3	ND	ND	ND
Feed containing 70 mg narasin /kg; 5 day withdrawal period.	1	ND	ND	ND
	2	ND	ND	ND
	3	ND	ND	ND

ND: Not detected
Limit of detection (LOD): 0.6 µg/kg
Limit of quantification (LOQ): 1.8 µg/kg

The narasin concentration in plasma was related to the concentration of narasin in the medicated feed. Plasma concentrations increased nearly 20 times when the narasin concentrations in feed were increased twenty times. In contrast, narasin concentrations in the muscle of chickens that were medicated with 70 mg narasin/kg feed increased only two-fold compared to chickens that were fed with 3.5 mg narasin/kg feed. While higher concentrations of narasin in medicated feed result in proportionally higher residue concentrations in plasma and muscle, the increase is not always a dose proportional increase in tissues. Narasin was not detected in plasma and muscle at the 3- and 5-day withdrawal periods indicating that narasin disappears rapidly from poultry tissues after the administration of the compound.

In the second study (Catherman, et al., 1991), 30 mature chicken hens (Single Comb White Leghorn) were housed individually in metabolism cages. ^{14}C-labelled narasin was injected via cardiac puncture (0.7 µCi in 100µl of 85% dimethyl sulfoxide and 15% saline as a vehicle). Blood samples were taken from 8 chickens at different hours post-injection from 0.5 to 18 h. Excreta were collected daily from individual hens. Groups of 6 chickens were killed by cervical dislocation on days 1, 7, 14 and 28 post-injection and were necropsied to recover liver, kidney, heart, ovary, fat, skin, bile and muscle.

Approximately 80% of the dose cleared from the plasma before the first blood sample was taken (0.5 h) and at 24 hours post-injection only trace amounts remained. Liver, heart, fat, skin and ovarian tissues contained traces of radioactivity 1 day post-injection. Muscle and kidney contained no detectable concentrations of ^{14}C on day 1. All organ tissues cleared the radiolabel by day 7 and no detectable radioactivity was present thereafter. In excreta, the highest amount of ^{14}C was founded on day 1 (49% of dose) and by day 13 there was no detectable radioactivity. Approximately 93.6% of the administered dose was eliminated in the excreta. The radioactivity is reported in Table 2.

Table 2: Activity and concentration of ^{14}C in excreta of chickens. [1, 2]

CHICKENS			
Day	n[3]	(% of dose)	(µg/kg)[4]
1	24	48.9 ± 3.4	725 ± 60
2	18	19.9 ± 2.8	371 ± 60
3	18	13.1 ± 2.1	163 ± 21
4	18	6.6 ± 1.1	66 ± 11
5	18	1.7 ± 0.4	26 ± 13
6	18	0.6 ± 0.6	4 ± 1
7	18	0.2 ± 0.1	2 ± 1
8	12	0.6 ± 0.2	5 ± 2
9	12	1.2 ± 0.5	12 ± 6
10	12	0.2 ± 0.1	2 ± 0.7
11	12	0.4 ± 0.1	3 ± 0.7
12	12	0.2 ± 1	1 ± 0.7
13	12	0	0
14	12	0	0

[1] Chicken was dosed with 0.7 µCi as narasin. Recovered radioactivity was assumed to remain associated with the narasin molecule.
Total excreta samples were collected daily.
[2] Values are ± S.E
[3] n= number of samples, each from an individual chicken.
[4] Narasin equivalents, micrograms per kilogram of excreta.

In the third study (Manthey, 1977a), 4 broilers chickens approximately eight weeks old and preconditioned to narasin at 80 mg/kg in feed, were each given a single oral capsule dose of ^{14}C-labelled narasin. Excreta were collected from each chicken daily (24 hour samples) and analysed for radiochemical content. More than 85 % of the dose was recovered within 48 hours.

Quail

In a non-GLP compliant study (Catherman, et al., 1991), 60 Japanese quail hens were randomly assigned to five groups of 12 hens each. The quails were injected with ^{14}C-labelled narasin via cardiac puncture (0.113 µCi in 50µl of 85% dimethyl sulfoxide and 15% saline as a vehicle). Blood samples were taken from 8 quails at different hours post-injection. Groups of 12 quails were killed by cervical dislocation on days 1, 7, 14 and 28 days post-injection and were necropsied to recover the liver, kidney, heart, ovary, fat, skin, bile and muscle. Excreta were collected daily (1 only at day 14). Approximately 92% of the dose cleared plasma before the first blood sample was taken (0.5 h) and at 24 hours post-injection only trace amounts remained. No detectable concentrations could be found at 7 days post-injection.

In the excreta, 68.2 % of ^{14}C was recovered on day 1 and 75% within 72 hours. Liver, heart, fat and ovarian tissues contained traces of radioactivity on 1 day post-injection. Muscle and kidney contained

no detectable concentrations of ^{14}C on day 1. All organ tissues cleared the radiolabel by day 7 and no detectable concentrations of ^{14}C narasin were present thereafter.

Cattle

A GLP compliant study (Manthey, et al., 1984a) was conducted to investigate the rate, route and quantitative nature of the excretion of ^{14}C-labelled narasin from 2 Hereford heifers. The cattle were acclimated to confinement in metabolism cages for approximately one week prior to dosing. To assure separation of urine from the faeces, animals were fitted with indwelling urethral catheters. Each heifer was given a single dose of ^{14}C narasin (about 11.0 μCi of radioactivity was placed singly in a gelatine capsule). Following dosing, the urine and faeces were collected quantitatively daily at about 24-hours intervals. A total of 93.4% and 80.1% of the administered radioactivity was recovered; up to 98% in the faeces and less than 0.5% in the urine. The radioactivity in the faeces was excreted within 4 days of dosing.

In a non-GLP compliant study (Manthey, at al., 1982), Hereford feedlot cattle (6 steers and 3 heifers) were dosed orally with an amount of ^{14}C-labelled narasin corresponding to narasin usage at about 19.8 mg/kg. The cattle were confined in metabolism cages and dosed each morning and evening for 3, 5 and 7 days. At 12 hours following the last dosing, the animals were slaughtered and muscle, back fat, kidney and liver were collected.

Liver contained the highest concentration of residues corresponding to 0.92, 0.74 and 0.84 mg narasin/kg equivalents from cattle dosed for 3, 5 and 7 days, respectively. Through one-way analysis of variance of the means, the liver residue values were not statistically different, indicating that steady-state equilibrium of total tissue residue was established within 3 days of dosing. In contrast, little more than trace concentrations of residues were found in the other tissues (0.006 and 0.03 mg/kg equivalents all days). In these tissues, the mean residue concentrations were not statistically different from all animals at all dosing periods. The residues did not reflect the duration of dosing, or differences in animal size or sex.

Pigs

Two GLP compliant studies were conducted to evaluate the pharmacokinetics of narasin in pigs.

In the first study (Sweeney, et. al., 1995), three groups of 4 pigs were fed ^{14}C-labelled narasin rations for 7 days at 30 mg/kg with zero withdrawal (treatment 1), 30 mg/kg with a three-day withdrawal (treatment 2) and 45 mg/kg with zero withdrawal (treatment 3). Urine and faeces were collected daily throughout the study. At slaughter, samples of liver, kidney, muscle, back fat, skin and bile were collected.

Radioactivity measurements showed that 3-5% of the recovered radioactivity was found in urine and 95-97% in the faeces. Liver was the edible tissue with the highest amount of residue for all treatment groups. The other tissues contained relatively little residue. The amounts of radioactive residues in the edible tissues are shown in Table 3.

Table 3: Summary of radiolabelled residues (mg/kg-equivalents) in the edible tissues of ^{14}C - narasin-treated pigs.

Treatment Group 1- 30 mg/kg- zero withdrawal					
Animal Number	Liver	Kidney	Muscle	Skin	Fat
71	0.63	0.04	ND	0.02	0.05
75	0.99	0.05	ND	0.07	0.11
77	0.60	0.04	ND	0.02	0.03
79	0.79	0.04	ND	0.04	0.06
Mean	0.75	0.04	ND	0.04	0.06
Treatment Group 2- 30 mg/kg- 3 days withdrawal					
Animal Number	Liver	Kidney	Muscle	Skin	Fat
64	0.18	0.01	ND	ND	0.02
69	0.18	0.02	ND	0.02	0.02
76	0.16	0.01	ND	ND	0.01
78	0.14	0.01	ND	0.01	0.02
Mean	0.17	0.01	ND	0.02	0.02
Treatment Group 3- 45 mg/kg- zero withdrawal					
Animal Number	Liver	Kidney	Muscle	Skin	Fat
63	1.19	0.09	0.02	0.04	0.09
70	1.80	0.09	0.01	0.04	0.08
72	0.96	0.07	ND	0.05	0.07
80	1.96	0.09	0.02	0.07	0.15
Mean	1.48	0.09	0.02	0.05	0.10

ND: No detectable residue based on mean of the control tissue cpm value plus three times the standard deviation.

In a second study (Donoho, et al., 1988), six crossbred pigs (4 males and 2 females) were fed a ration containing ^{14}C-labelled narasin at a concentration equivalent to 37.5 mg/kg. The pigs were placed into separate metabolism crates. Three pigs (2 males and 1 female) were fed for 9 days and a similar group was fed for 5 days. All of the animals were killed at 12 hours after the last dose.
The mean liver radioactivity concentration at zero withdrawal was 0.51 mg/kg equivalents for the pigs dosed for 5 days and 0.55 mg/kg for the pigs dosed for 9 days. There was no statistical difference between the two groups indicating that 5 days was an adequate period to establish steady-state concentrations. In one male pig, urine and faeces were collected daily. Approximately 6-8% of the administered dose was recovered in urine and 92-94% was recovered in the faeces.

Metabolism in Laboratory Animals

Rats

In a GLP compliant study (Sweeney and Kennington, 1994), 10 male and 10 female Fischer strain 344 rats were given daily oral (gavage) doses of 5 mg narasin/kg bw for 5 days. Urine and faeces were collected daily from all animals and extracted for metabolite profiling. Narasin metabolites in the extract were identified by high performance liquid chromatography/ion spray-mass spectrometry (HPLC/ISP-MS).

In the faeces, four structural isomers of tri-hydroxy narasin A and four di-hydroxy narasin A were identified. Four peaks were identified as tri-hydroxy narasin B and four as di-hydroxynarasin B. Using high performance liquid chromatography/ion spray-mass spectrometry (HPLC/ISP/MS), the

exact position of hydroxylation could not be determined in this study. These metabolites demonstrate that the narasin metabolic pathways in the rat include hydroxylation and oxidation.

In another GLP compliant study (Manthey and Goebel, 1986), 6 mature rats, 3 males and 3 females, were placed in individual metabolism cages. The rats were dosed by gavage for 5 consecutive days. Each dose was 1 mL of the acacia ^{14}C -narasin suspension, which corresponded to a dose of about 3.3 mg ^{14}C-narasin/kg bw. Faeces were collected daily during the dosing period. About four hours after the last dose, the rats were killed, necropsied and the livers were collected immediately. In this study, ^{14}C-narasin was metabolized to more than twenty metabolites and the pattern in faeces and liver was qualitatively similar.

Dogs

In a GLP compliant study (Manthey and Goebel, 1986), a mature male dog, weighing 11.8 kg, was placed in a metabolism cage and acclimated for 4 days prior to dosing. This study was conducted to make a comparison between cattle, rat and dog. The animal was dosed by oral gavage for 4 consecutive days; one-half of the allotted dose was given in the morning and the other half at mid-day. The dose was 2.0 mg ^{14}C-narasin/kg bw. The faeces were collected each day and stored in a freezer during the dosing period. Urine was not collected. About 4 hours after the last dose, the dog was euthanized by injection of sodium phenobarbital. The liver was excised immediately, chopped and frozen. The study demonstrated that ^{14}C-narasin is metabolized to more than 20 metabolites by those species. No single metabolite accounts for a large proportion of the total. The pattern of narasin metabolites in faeces and liver is qualitatively similar among the three species, although there are quantitative differences. The primary metabolic pathway appears to be oxidation (hydroxylation) of the narasin at various sites on the polyether rings. The metabolites that have been identified are mono- di- or tri-hydroxy narasin derivates.

Metabolism in Food Producing Animals

Cattle

As noted above, a GLP compliant study (Manthey and Goebel, 1986) was conducted to compare the metabolism of ^{14}C narasin in orally dosed cattle (target animal), dog and rats. This study indicated that the pattern of narasin metabolites in faeces and liver was qualitatively similar among the three species, although there were quantitative differences. Liver is the only edible tissue in cattle that contains appreciable concentrations of residue. The most abundant metabolite in cattle liver is NM-12, a mono-hydroxy narasin, which accounts for approximately 15% of the liver radioactivity. Metabolite NM-13 (di-hydroxy narasin) is relatively abundant in cattle faeces.

Pigs

In a GLP compliant study (Sweeney, et. al., 1995), three groups of 4 pigs were fed ^{14}C-narasin rations for 7 days containing 30 mg narasin/kg with zero withdrawal (treatment 1), 30 mg narasin/kg with three day withdrawal (treatment 2) and 45 mg narasin/kg with zero withdrawal (treatment 3). Pigs were individually housed in metabolism cages. Urine and faeces were collected from each animal daily throughout the study. Pigs were slaughtered by captive bolt and exsanguination and samples of liver, kidney, skin, muscle, back fat and bile were collected.

Narasin metabolites were characterized using high performance liquid chromatography/electrospray-mass spectrometry/liquid scintillation counting (LC/EPS-MS/LSC). Liver contained the greatest amount of residue in all treatment groups; other tissues containing relatively low residues. The mean concentration of radioactivity in all tissues was greater in pigs fed 45 mg narasin/kg than those fed 30 mg narasin/kg at zero withdrawal. In the group that was fed with 30 mg/kg after a three-day withdrawal, the total residues in each tissue had depleted to one-fourth in liver and kidney, to one one-half in skin and to one third in fat of the concentrations at zero withdrawal, respectively. A number of hydroxylated metabolites of narasin and narasin B were identified in the liver, bile and faeces at zero withdrawal. The total ion chromatograms, radiochromatograms and mass spectra for bile and faeces

are similar to those seen for liver. Five hydroxylated metabolites were identified as being common to both faeces and liver. The metabolic profile of narasin in liver, bile and faeces is summarized in Tables 4, 5 and 6. These data show that narasin is extensively metabolized by pigs and hydroxylation is the main metabolic pathway in liver, bile and faeces.

Table 4: Metabolites in pig liver from TIC (Total Ion Chromatogram) and radiochromatogram in pigs fed 30 mg narasin/kg with zero withdrawal.

Metabolite ID	TICpeak	RC* peak	Ammoniated/Sodiated molecular ion	% Injected Radioactivity	Proposed structure*
N-1	A	1	828/833, 830/835	4.3	OH3B,OH3
N-3	B	2	828/833, 830/835	5.2	OH3B,OH3
N-4, N-5	C	3	812/817	10.3	OH2B
	D	4	812/817	4.4	OH2B
N7	E	5	814/819	3.8	OH2
	F	6	814/819	3.0	OH2
	G	7	814/819	6.2	OH2
	H	8	814/819	2.2	OH2
	I	9	798/803	1.2	OH
			% Total Injected Radioactivity	40.6	

Table 5: Metabolites in pig bile from TIC (Total Ion Chromatogram) and radiochromatogram in pigs fed 30 mg narasin/kg with zero withdrawal.

TICpeak	RC* peak	Ammoniated/Sodiated Molecular ion	% Injected Radioactivity	Proposed structure*
A	1	830/835	4.1	OH3
B	2	830/835, 828/833	14.4	OH3, OH3B
C	3	830/835	4.6	OH3
D	4	814/819,812/817	13.6	OH2, OH2B
E	5	812/817	9.4	OH2B
F	6	812/817	3.4	OH2B
G	7	814/819	2.7	OH2
H	8	814/819	5.5	OH2
I	9	814/819	5.1	OH2
% Total Injected Radioactivity			62.8	

Table 6: Metabolites in pig faeces from TIC (Total Ion Chromatogram) and radiochromatogram in pigs fed with 30 mg narasin/kg with zero withdrawal.

Metabolite ID	TIC peak	RC* peak	Ammoniated/Sodiated molecular ion	% Injected Radioactivity	Proposed structure*
N-2	A	1	830/835	3.3	OH3
N-3	B	2	828/833	4.4	OH3B
	C	3	812/817	4.8	OH2B
N-4	D	4	812/817	20.9	OH2B
N-5	E	5	812/817	4.1	OH2B
N-6	F	6	814/819	3.8	OH2
	G	7	814/819	1.3	OH2
	H	8	812/817	6.5	OH2B
N-7	I	9	814/819	5.3	OH2
	J	10	814/819	2.1	OH2
	K	11	814/819	1.6	OH2
	L	12	814/819	2.2	OH2
	M	13	782/787	3.8	Narasin
	N	14	780/785	0.3	Narasin B
% Total Injected Radioactivity				64.6	

* OH2 = di-hydroxynarasin, OH3 = tri-hydroxynarasin

Chickens

Two GLP compliant studies were conducted to evaluate the metabolism of narasin in chickens. In the first study (Holmstrom, et al., 2002), the metabolic fate of ^{14}C -narasin in the edible tissues and excreta of 20 broiler chickens was studied at practical zero withdrawal (6 hour) following 5 consecutive days of treatment with medicated feed provided *ad libitum*. The feed contained a nominal 80 mg narasin/kg (71.1 mg narasin/kg measured as narasin A). The animals were housed in individual stainless steel cages in a temperature controlled environment. Six control broilers received un-medicated feed. Excreta were collected daily beginning one day prior to dosing until the day of slaughter. Livers were collected at necropsy.

Extensive metabolism of narasin A was noted in the liver with oxidative hydroxylation as the primary pathway of metabolism. The predominant metabolites are di-hydroxylated and tri-hydroxylated narasin A, representing 42% of total radioactivity injected. Narasin A metabolites identified in excreta included hydroxylated, di-hydroxylated and tri-hydroxylated narasin A and di and tri-hydroxylated analogs of an oxidized form due to ketone formation. These metabolites represented 88.9% of total radioactivity injected. Identified metabolites and their respective calculated concentrations in liver and excreta are shown in Table 7.

Table 7: Quantification of metabolites in liver and excreta by radiochromatograms collected concomitantly using mass spectrometry.

Liver			
Metabolite ID	Proposed metabolite structure	Percent of total Radioactivity Injected	Estimated Concentration[1] in Liver, mg/kg
NL3	Trihydroxynarasin A	16	0.04
NL1,NL2	Dihydroxynarasin A	8	0.02
NL4	Trihydroxynarasin A	18	0.05
Total		42	0.12
Excreta			
Metabolite ID	Proposed metabolite structure	Percent of total Radioactivity Injected	Estimated Concentration[1] in Liver, mg/kg
NE6	Trihydroxynarasin A	6.9	4.9
NE7	Trihydroxynarasin A	19.6	13.9
NE8	Trihydroxynarasin A	6.2	4.4
NE10	9-Keto-trihydroxynarasin A	6.2	4.4
NE3	Dihydroxynarasin A	2.9	2.1
NE1 NE9 NE12	Hydroxynarasin A Trihydroxynarasin A Trihydroxynarasin A	3.7	2.6
NE4 NE11	Dihydroxynarasin A 9-Keto-trihydroxynarasin A	34.4	24.3
E5	9-Keto-dihydroxynarasin A	8.6	6.1
 NE2	Narasin A Hydroxynarasin A	0.4	0.3
Total		88.9	62.8

[1]Calculated by multiplying mean residue concentration by fraction of total radioactivity injected.

In the second study (Sweeney, et al., 1994), 5 chickens were fed rations containing 50 mg ^{14}C narasin/kg feed. Excreta were collected from each pen beginning 1 day before initiation of the study and continuing until the end of treatment. After 5 days, the chickens were slaughtered and samples of liver, kidney, muscle, fat and skin/fat were collected and assayed for total radioactivity by solubilisation and liquid scintillation counting.

Liver was the tissue with highest concentration of extractable radioactivity (61%) but individual metabolites could not be identified because of the low amount of radioactivity in the liver. Kidney and muscle had a mean concentration ≤0.05 mg/kg and fat, skin/fat ≤ 0.12 mg/kg. At least fifteen metabolites and parent narasin were identified from the excreta. These metabolites were predominately di and tri-hydroxylated narasin A and di and tri-hydroxylated narasin B. The distribution and relative magnitude of radioactivity from liver and excreta were similar, suggesting that excreta metabolites are the same as those found in liver. The results and indicated molecular ions for each metabolite in excreta are shown in Table 8.

Table 8: Narasin metabolites characterized in excreta. Peak determined from overlay of TIC* on the radiochromatogram.

TIC peak number	Radio chromatogram peak number	Ammoniated/Sodiated Molecular Ion	% Total radioactivity	Proposed structure
	1		1.0	Tetrahydroxynarasin
A	2	846/851	2.4	Trihydroxynarasin
B	3	830/835	13.4	Trihydroxynarasin
C	3	830/835	**	Trihydroxynarasin B
D	4	828/833	6.9	Trihydroxynarasin B
E	4	828/833	**	Trihydroxynarasin B
F	4	828/833	**	Trihydroxynarasin B
G	5	828/833	3.0	Trihydroxynarasin B
H	5	828/833	**	Trihydroxynarasin B
I	6	812/817	6.2	Dihydroxynarasin B
J	7	814/819	4.4	Dihydroxynarasin
K	7	814/819	**	Dihydroxynarasin
L	8	812/817	1.9	Dihydroxynarasin B
M	9	814/819-812/817	1.88	Dihydroxynarasin /Dihydroxynarasin B
N	10	814/819-828/833	1.86	Dihydroxynarasin /Trihydroxynarasin B
O	11	828/833	0.48	Trihydroxynarasin B
% of Total Radioactivity			43.46	

In a non-GLP compliant study, six metabolites of narasin were isolated from excreta of chickens that were fed a ration containing 100 mg ^{14}C narasin/kg. Four metabolites were tentatively identified as di-hydroxynarasin and two as tri-hydroxynarasin. The six metabolites were assayed for antimicrobial activity against *Bacillus subtilis* in a standard narasin TLC bioautographic assay system. These metabolites were 20 times less active than narasin (Manthey and Goebel, 1982).

TISSUE RESIDUE DEPLETION STUDIES

Radiolabelled Residue Depletion Studies

Cattle

In a GLP compliant study (Manthey, et al., 1984b), Hereford feedlot cattle, 6 steers and 3 heifers, naïve to narasin and weighing between 185-220 kg, were used as test and control animals. The cattle were confined in individual metabolism cages located in a temperature-controlled barn. Each animal received a single capsule with ^{14}C narasin equivalent to 13 mg/kg feed administered orally using a bolus gun. The animals were dosed morning and evening for 5 consecutive days. At each of the withdrawal times of zero (12 hours after the final capsule dose), 1 and 3 days, cattle were killed. Samples of liver, kidney and back fat were collected immediately for radiochemical analysis. The mean net radiochemical residues were calculated as mg narasin/kg equivalents.

Liver contained the highest concentrations of radioactivity corresponding to 0.49, 0.23 and 0.05 mg narasin /kg equivalents at the withdrawal times of zero, 1 and 3 days, respectively. Less than 5% of the liver radioactivity corresponded to parent narasin. Muscle, fat and kidney contained less than 0.02 mg narasin/kg equivalents at zero withdrawal. Results are provided in Table 9.

Table 9: Mean net[a] radioactivity in tissues of cattle following oral dosing with ^{14}C-narasin at a concentration equivalent to a 13 mg/kg ration.

Tissue radioactivity as mg/kg Narasin equivalents						
Animal Number	Sex	Days Withdrawal	Liver	Kidney	Back Fat	Muscle
915	F	0	0.49	0.01	0.02	0.003
871	F	0	0.39	0.01	0.01	0.006
862	M	0	0.60	0.1	0.02	NNR[b]
Mean			0.49	0.01	0.02	
916	F	1	0.19	0.002	0.003	0.002
876	M	1	0.28	0.002	0.009	0.002
867	M	1	0.23	0.004	0.002	NNR[b]
Mean			0.23	0.003	0.005	
914	F	3	0.04	NNR[b]	0.001	NNR[b]
905	M	3	0.05	NNR[b]	NNR[b]	0.004
861	M	3	0.07	NNR[b]	0.001	0.002
Mean			0.05			

a) Net mg/kg equivalent to: net dpm/g ÷779 dpm/µg
b) No net residue. Negative net values were derived for these samples

Pigs

In a GLP compliant study (Donoho, et al., 1988), pigs (male and female) weighing approximately 22 kg, were fed a ration containing ^{14}C-narasin equivalent to 37.5 mg/kg for 5 days. Half of the daily dose was given in the morning and the other half in the evening. Groups of 3 pigs were killed at 0 (12 hours after the last dose), 24, 48 or 72 hours withdrawal time. Muscle, liver, kidney, skin and fat were assayed for total radioactivity. Total radioactivity in liver for 0, 24, 48 and 72 h withdrawal were 0.51, 0.44, 0.26 and 0.18 mg/kg-equivalents, respectively. Muscle and kidney contained no radioactivity at zero withdrawal and fat contained less than 0.05 mg/kg equivalents of narasin. Other withdrawal times were not assayed because zero residues were of no practical significance. The results are showed in Table 10.

Table 10: Radioactivity concentrations of narasin in tissues of pigs.

Net Radioactivity (mg narasin equivalents/kg)						
Animal Number and Sex	Dosing Period	Withdrawal Time (hours)	Liver	Muscle	Kidney	Fat
H136859-M	5 day	Zero	0.37	NDR	NDR	NDR
H136890-M	5 day	Zero	0.42	NDR	NDR	NDR
H136896-F	5 day	Zero	0.74	NDR	NDR	0.04
		mean ± s.d	0.51 ± 0.2			
H131886-M	5 day	24 hrs.	0.49	-	-	-
H131882-M	5 day	24 hrs.	0.43	-	-	-
H131876-F	5 day	24 hrs.	0.40	-	-	-
		mean ± s.d	0.44 ± 0.04			
H131880-M	5 day	48 hrs.	0.28	-	-	-
H131881-M	5 day	48 hrs.	0.24	-	-	-
H131884-F	5 day	48 hrs.	0.27	-	-	-
		mean ± s.d	0.26 ± 0.02			
H131878-M	5 day	72 hrs.	0.18	-	-	-
H131879-M	5 day	72 hrs.	0.19	-	-	-
H131885-F	5 day	72 hrs.	0.18	-	-	-
		mean ± s.d	0.18 ± 0.01			

NDR: No detectable residue.

Chickens/Turkeys

In a non-GLP compliant study (Manthey, et al., 1983), male and female chickens were grown from one day of age using a nominal 80 mg narasin/kg ration. At about eight weeks of age the birds were dosed with 80 mg ^{14}C-narasin (1.35 or 1.01 µCi/mg)/kg ration *ad libitum* for 5 days and then slaughtered at zero, 1 and 3 days of withdrawal. Muscle, liver, kidney, skin and fat were assayed for total radioactivity. Radioactivity concentration in tissues was presented as mg narasin/kg-equivalents. Liver contained the highest ^{14}C-residues and muscle contained the lowest. At 3 days withdrawal all residues were below 0.025 mg/kg equivalents with the exception of liver, which was approximately 0.07 mg/kg equivalents.

In a non GLP compliant study (Manthey, 1977b), 12 broilers chickens were grown for eight weeks on feed that contained 80 mg narasin/kg. The chickens then received capsule doses of ^{14}C-narasin, each of which contained 4.6mg (0.297 µCi/mg ^{14}C-narasin) orally morning and evening for two and one-half days. During this period and the withdrawal periods, the chickens were maintained on non-medicated feed. Withdrawal times were zero (four hours after the last dose), 1, 2, 3, 5 and 7 days. One male and one female were sacrificed at each withdrawal time and muscle, liver, kidney and fat tissues and skin were collected.

At zero withdrawal time, radiochemical residues were found in all tissues except muscle. Liver contained the highest residue concentration, which represented 0.50 mg narasin/kg equivalents. After two days withdrawal, the concentration declined by 93% and no residue exceeded 0.04 µg narasin/kg equivalents. The tissue residues declined progressively throughout the withdrawal period to negligible concentrations. The results are shown in Table 11.

Table 11: Net radiochemical residues as mg narasin/kg-equivalents in tissues of chickens treated orally with ^{14}C-narasin.

Withdrawal Time (days)	Sex	Muscle	Liver	Kidney	Fat	Skin
Zero	M	ND[1]	0.01	ND	0.04	0.05
	F	ND	0.50	0.11	0.22	0.17
1	M	ND	0.13	ND	ND	0.06
	F	ND	0.12	ND	0.13	0.08
2	M	[2]	ND	ND	ND	0.02
	F		0.04	ND	ND	0.04
3	M	[2]	ND	ND	ND	0.03
	F		0.04	ND	ND	0.02
5	M	[2]	ND	ND	ND	0.02
	F		ND	ND	ND	0.00

[1] No net residue exceeded the 95% upper confidence limit of control mean
[2] Not assayed

In a GLP compliant study (Manthey, et al., 1981), broiler chickens approximately seven week of age were dosed for 5 days with a broiler ration containing 100 mg ^{14}C narasin/kg. At each of five withdrawal intervals, zero, 1, 2, 3 and 5 days, three birds (two male and one female) were sacrificed. Muscle liver, kidney, fat, muscle and skin samples were taken from each chicken. Total radioactivity was determined by combustion analysis and scintillation counting and the mean net radiochemical residues were calculated as mg narasin/kg equivalents.

The zero withdrawal time values of narasin in mg/kg equivalents were: liver, 0.45; fat, 0.21; skin, 0.14; kidney, 0.14; and muscle, 0.02. Following withdrawal of medication, the radiochemical residues declined sharply in all tissues. After a 1 day withdrawal, the residue concentrations had declined by more than 50 percent and all tissues except liver were below 0.1 mg narasin/kg equivalents. A summary of these data is given in Table 12.

Table 12: Net[1] radioactivity as mg narasin/kg equivalents in chickens fed 100 mg ^{14}C narasin/kg feed.

Withdrawal Time (days)	Mean values mg/kg equivalents (n=3)				
	Muscle	Liver	Kidney	Fat	Skin
Zero	0.02	0.45	0.14	0.21	0.14
1	0.01	0.18	0.05	0.06	0.06
2	0.01	0.12	0.03	0.02	0.02
3	0.01	0.13	0.03	0.01	0.03
3	0.01	0.10	0.02	0.01	0.03

[1] Net mg/kg-equivalent to: (gross dpm/g – control dpm/g) ÷ 932 dpm/µg

Residue Depletion Studies with Unlabelled Drug

Residues in Tissues

Cattle

In a non-GLP compliant study (Potter and Cooley, 1975), the residue pattern over different withdrawal times (0, 24, 48 and 120 h) was determined using a TLC-bio-autographic method. Eighteen Hereford cattle were allotted by weight to four treatment groups. The animals were fed 150 mg narasin/head/day (65 mg narasin/kg) for 140 days. At the time of slaughter, representative samples

of muscle, fat, liver and kidney were collected. The results showed concentrations less than 5 µg/kg of narasin in the muscle tissue at zero withdrawal and no residues were found at subsequent sampling times. Residues were found in the fat and liver up to 48 hrs withdrawal (10 µg/kg and less). No residues were found in kidney at any time.

Pigs

In a non-GLP compliant study, (Moran et al., 1992) the concentrations of narasin residues were determined in the muscle and liver tissues of pigs (12 barrows and 12 female) fed with a finishing ration containing 0 or 45 mg narasin/kg *ad libitum* for 14 days. The animals were assigned to four pens with equal numbers of each sex. Tissues were collected at 12 and 24 hours withdrawal time and were analyzed for the presence of narasin. No residues at or above the limit of quantification of the method (LOQ = 25 µg/kg) were observed in the tissues of any animals sacrificed at either hours.

Chickens

Three GLP compliant studies were conducted to evaluate residues of unlabelled narasin in the edible tissues of chickens.

In the first study (Lacoste and Larvor, 2003), 32 Ross broilers chickens (an equal number of male and females) were fed 80 mg narasin/kg feed for 5 consecutive days. Birds were housed in communal cages on a slatted wire floor in groups of four (assigned in cages by sex). Birds were slaughtered and tissue samples were taken at 0, 6, 12, and 24 h withdrawal time. Narasin was quantified by HPLC with UV detection after post-column derivatization. The limit of quantification (LOQ) was 25 µg/kg and limit of detection (LOD) was 10 µg/kg. Narasin was not detected at zero h withdrawal time in muscle and kidney. In liver and in skin/fat, narasin was not detected at 6 hours and 24 hours withdrawal time. The results are represented in Table 13.

Table 13: Narasin residues in chicken tissues.

		Mean concentration (µg/kg)			
Withdrawal Time (hours)	Number of. Chickens	Muscle	Liver	Kidney	Skin/Fat
0	8	ND	46.2	BLQ	67.1
6	8	ND	ND	ND	39.1
12	8	ND	ND	ND	BLQ
24.	8	ND	ND	ND	ND

BLQ: Below limit of quantification
ND: Not detected

In the second study (Maruyama and Sugimoto, 2000), broiler chickens were fed a medicated ration from day 0 to day 42. Three groups of birds were used, one control group (non-medicated feed), the second group was fed with medicated feed containing 80 mg narasin/kg and the third group fed with medicated feed containing 160 mg narasin/kg. Nine chickens per group were slaughtered by exsanguination. Tissue samples were taken at day 21 during medicated feed administration and at 42 days, at 2, 24, 72, 120 and 168 hours withdrawal. Muscle, liver, kidney skin and fat samples were taken. Narasin residues were determined by bio-autography using *Bacillus stearothermophilus* var. *calidolactis* C-953 as the indicator organism (Limit of quantification = 25 µg/kg).

In the 80 mg narasin/kg dose group, narasin residues were quantified in fat and skin at 2 and 24 hours withdrawal time, respectively. In the other tissues, there were no quantifiable narasin residues in any of the withdrawal times. The results are shown in Table 14. In the 160 mg narasin/kg dose group, narasin residues were quantified in higher concentrations in fat and skin at 2 hours withdrawal. In all

tissues, narasin was not quantified at 24 hours with the exception of skin (72 hours). The results are shown in Table 14.

Table 14: Residues in chicken tissues (mg/kg) using 80 mg/kg medicated feed.

Test Groups	Sampling Point	Sample No.	Muscle	Liver	Kidney	Fat	Skin
NARASIN 80mg/kg	Day 21	1	<0.025	<0.025	<0.025	0.15	0.09
		2	<0.025	<0.025	<0.025	0.14	0.15
		3	<0.025	<0.025	<0.025	0.09	0.17
		Average				0.13	0.13
	2 hours[1]	4	<0.025	<0.025	<0.025	0.09	0.05
		5	<0.025	<0.025	<0.025	0.06	0.03
		6	<0.025	<0.025	<0.025	0.13	0.04
		Average				0.09	0.04
	24 hours[1]	7	<0.025	<0.025	<0.025	<0.025	<0.025
		8	<0.025	<0.025	<0.025	<0.025	0.03
		9	<0.025	<0.025	<0.025	<0.025	0.03
	72 hours[1]	10				<0.025	<0.025
		11				<0.025	<0.025
		12				<0.025	<0.025
	120 hours[1]	13					<0.025
		14					<0.025
		15					<0.025

[1] Samples times post treatment at 42 days

Table 15: Residues in chicken tissues (mg/kg) using 160 mg/kg medicated feed.

Test Group	Sampling Point	Sample No.	Muscle	Liver	Kidney	Fat	Skin
NARASIN 160mg/kg	Day 21	21	<0.025	<0.025	<0.025	0.21	0.51
		22	<0.025	0.029	<0.025	0.20	0.47
		23	<0.025	0.026	<0.025	0.20	0.35
		Average				0.20	0.44
	2 hours[1]	24	<0.025	<0.025	<0.025	0.19	0.10
		25	<0.025	<0.025	<0.025	0.12	0.07
		26	<0.025	<0.025	<0.025	0.17	0.09
		Average				0.16	0.08
	24 hours[1]	27	<0.025	<0.025	<0.025	<0.025	<0.025
		28	<0.025	<0.025	<0.025	<0.025	<0.025
		29	<0.025	<0.025	<0.025	<0.025	0.032
	72 hours[1]	30				<0.025	<0.025
		31				<0.025	<0.025
		32				<0.025	<0.025
	120 hours[1]	33					<0.025
		34					<0.025
							<0.025

[1] Samples times post treatment at 42 days

In the third study (Handy, et al., 1985), one day-old Hubbard X White Mountain broiler chicks were fed for at least 45 days with a ration containing 80 mg narasin/kg. Four male and four female birds were slaughtered at each sampling time. Skin with adhering fat and abdominal fat tissue samples were

collected after 6, 12, 18 and 28 hours withdrawal time. The analyses were realized by bio-autographic assay using *Bacillus subtilis* as the indicator organism. The limit of detection was 5 µg/kg. Concentrations above the limit of detection were found up to 28 hours withdrawal time. No statistical differences in residue concentration due to sex were observed.

METHODS OF ANALYSIS

For detection of narasin residues different methods have been described.

Screening methods

In a GLP compliant study (Maruyama and Sugimoto, 2000), screening by thin layer chromatography - bio-autography has been developed. The extraction procedure for tissue samples is based on solvent extraction with acetonitrile and n-propanol and further purification using a Sep-Pack silica cartridge. The bio-autography was performed by melting agar over the surface of the TLC plate seeded with *Bacillus stearothermophilus* var. *calidolactis* C-953 innoculum. After incubation for 18 hours at 56 °C, the zones of inhibition were measured to determine narasin presence. The limit of quantification (LOQ) was estimated considering 2.0g of sample, 0.5ml final volume of sample solution and minimal concentration of standard solution of 0.1µg/mL.. The LOQ was 25 µg/kg: Recovery from tissues was tested by the addition of 0.4 µg of narasin standard to the 2.0 g the control tissues. At this concentration, the recoveries were 84 – 100 %. The authors reported that the calibration curves showed good linearity within the tested concentrations of 0.1 - 3.2mg/kg. The accuracy, precision and the limit of detection (LOD) of the assay were not given.

In another GLP compliant screening study (Handy, et al, 1985), a TLC-bio-autographic method, using *Bacillus subtilis* as the indicator organism, was described. For this method, the limit of detection was 5 µg/kg.

A Time-Resolved Fluorescence Immunoassay (TR-FIA) screening method for the detection of narasin was developed in a non - GLP compliant study (Peippo et al., 2004). With this method, the muscle samples were treated with acetonitrile and the clean up was accomplished with an SPE silica cartridge. The eluate was reduced to dryness under nitrogen stream and reconstituted in a buffer. The resulting solution was applied to a microtiter well containing the antibody (goat anti-sheep IgG), and an aliquot of unlabelled narasin-transferrin conjugate in a reconstitution buffer was added. The plates were washed with wash solution and finally an enhancement solution was added to each plate. The time resolved fluorescence was measured by a multi-label counter. The LOD of this method was 560 µg/kg, the LOQ was 800 µg/kg. The results of the precision intra-assay and inter-assay were 3.5 and 3.6% (CV) respectively. The recovery for narasin was 89.6% with a CV of 4.1%.

Confirmatory methods

There are different published of HPLC and mass spectrometric methods to determinate narasin in the edible tissues of chickens:

HPLC methods with UV vis detection:

For these analyses, the extractions of the samples are performed with solvent and the purification is performed with a silica SPE cartridge. The sample is dried by a nitrogen stream, dissolved with a diluent solvent and transferred into a HPLC vial for analysis. The chromatographic analysis uses postcolumn derivatization with vanillin reagent, which produces a colored product that absorbs at 520 nm. (Ward et al., 2005; Lacoste and Larvor, 2003) In Table 16, the performance data are summarized.

Table 16: Performance data for the HPLC methods with UV vis detection.

Criteria	Lacoste and Larvor, 2003	Ward, et. al, 2005
QA System	GLP	In house
Matrices	Skin/fat, muscle, liver, kidney	Skin/fat, muscle, liver, kidney
LOQ	25 µg/kg	7 µg/kg
LOD	10 µg/kg	3 µg/kg
Linearity	-----	0.9995 - 0.9999
Calibration curve range	5 - 50 µg/mL	0.125 - 1.0 µg/mL
Recovery %	77.5 – 80.6	76.0 - 92.6
Repeatability (C.V %)	4.1 - 6.5	-
Reproducibility		
Ruggedness testing		
Confirmatory method	None	None

Mass spectrometric methods:

Different authors have described the use of LC coupled to mass spectrometry to determine narasin in edible broiler tissues. The method included a short sample extraction and a minimal sample purification procedure. The tissues are treated with anhydrous sodium sulphate and extracted with acetonitrile and the clean up is performed with a silica SPE cartridge. The eluate is taken to dryness using nitrogen flow and then is redissolved in acetonitrile and ammonium acetate and transferred into a vial for HPLC/MS/MS analysis. The analyses are performed in the positive ion electrospray modes. The parent ion is 787, and the transitions used for the narasin confirmation are 787>431 and the 787>531. In table 17, the performance criteria of the mass spectrometric methods are shown.

Table 17: Performance criteria mass spectrometric methods.

Criteria	Rokka and Peltonen, 2006	Matabudul, et al., 2002	Dubois, et al., 2004
QA system	In house	In house	In house
Matrices	Muscle	Liver and eggs	Muscle and eggs
LOQ:		1 µg/kg	
LOD:	1 µg/kg		
CCα*	1.6 µg/kg		0.3 µg/kg
CCβ*	1.9 µg/kg		0.4 µg/kg
Linearity (r^2)	> 0.990	0.99	
Calibration curve range	1 - 5 µg/kg	1 - 50 µg/kg	
Recovery %	63 – 70	93 - 118	53
Repeatability (CV %)	5.3 - 7.0		
Reproducibility (CV %)	12 – 27	6.3 - 13.7	
Ruggedness testing	Not reported	Not reported	Not reported
Confirmatory method	Yes	Yes	Yes

CCα: Decision Limit, CCβ: Decision Capability

The mass spectrometric methods are suitable and provide better specificity (without interference signals around the retention time) and sensitivity than do the HPLC-UV methods. Furthermore, because the methods require only a simple extraction with a short run time (about 12 min), large samples batches (more than 20 samples) can be processed daily.

APPRAISAL

Narasin has not been previously reviewed by the Committee. It is a polyether monocarboxylic acid ionophore. It is composed of the analogues A, B, D and I. Narasin A is the major component (equivalent to 96%) and it has at least 85% of the activity. It has been classified as an anticoccidial drug in veterinary medicine and is intended to prevent and control coccidiosis caused by *Eimeria* in broilers chickens. Narasin is used at a dose range of 54 – 72 mg narasin/kg in complete feed.

Pharmacokinetics studies in both target and laboratory animals show that orally administered narasin is rapidly metabolised and eliminated within a few days. Eighty-five percent of the dose is detected in the excreta within 48 hours. Radioactivity collected from the excreta of rats and chickens shows that a low percentage (3-5%) of the recovered radioactivity is in urine and over 90% in the faeces.

Metabolism was studied in animals using ^{14}C-radiolabelled narasin. In those studies, multiple metabolites of narasin A and narasin B have been identified in excreta. Unchanged narasin represented less than 3% of the total radioactivity. Liver metabolites are the same as those found in excreta. Hydroxylation appears to be the major route for the metabolism of narasin to polar inactive metabolites. Comparative studies indicate that the metabolite pattern is qualitatively similar among species; however there are quantitative differences. Antimicrobial activity studies against *Bacillus subtilis* indicate that hydroxylated metabolites have at least twenty times less activity than narasin A.

The radiolabelled and unlabelled depletion studies in chickens using different doses of narasin in feed and different dosing periods show that this drug is quickly metabolized and narasin disappears very rapidly from tissue. The major concentrations up to 6 hour withdrawal periods are detected in liver. At 2 hours withdrawal, residues are not detected in muscle and kidney; residues can be detected in skin/fat up to 24 hours withdrawal.

The liver is suitable as the target tissue, but for residue control purposes skin/fat also may be considered. Parent narasin is the appropriate marker residue because it is present in nearly all the edible tissues. Narasin metabolites have little or no microbiological activity *in vitro.*

Suitable analytical methods have been described for the determination and confirmation of narasin in edible tissues of chickens and pigs. These methods include HPLC with UV detection (LOQ of 25µg/kg wet tissues) that could be using for monitoring residues of narasin A in different tissues. Confirmatory methods such as HPLC/MS/MS provide good specificity and sensitivity. The monitoring of two parent-daughter transitions are enough to confirm the presence of presumptive positives for narasin residues. The calibration curve ranges of these methods present good linearity ($r^2 \geq 0.99$) and each point differs no more than 100 ± 10% of the mean of the response/concentration. For the HPLC/MS/MS method, an LOQ of 1 µg/kg wet tissues and a CCα of 0.3 and 1.6 µg/kg wet tissues have been described.

Residues in cattle may be determined using a TLC-bioautographic method. This method, while having a reported test sensitivity of 5 µg/kg, however, reports residue values only as a range (*e.g.*, 10-20; 5-10). As a result, in recommending permanent MRLs for pigs and chickens and temporary MRLs for cattle the Committee used the LOQ values for the HPLC-UV method.

MAXIMUM RESIDUE LIMITS

In recommending MRLs for narasin in chickens and pigs and temporary MRLs for cattle, the Committee considered the following factors:

- An ADI of 0-5 µg/kg bw was established by the Committee based on a toxicological endpoint. This ADI is equivalent to up to 300 µg for a 60 kg person.
- Narasin A is a suitable marker residue in tissue.
- Metabolites exhibit little or no microbiological activity *in vitro*. Unchanged narasin represents approximately 5% of the total residues in liver.

- Liver contains the highest concentrations of residues. In fat, narasin residues persist for up to 72 h. Liver or fat (skin/fat in natural proportion, where applicable) are considered suitable choices for the target tissue.
- Residue data in the studies submitted were determined using several methods. These methods include a validated HPLC with post-column derivatization and UV detection and a validated HPLC/MS/MS. Both of these newer methods are suitable for routine monitoring.
- The analytical methods have been validated for chicken and pig tissues. The methods have not been adequately validated for cattle tissues.
- Because residue concentrations in chickens and pigs were low or non-detectable beyond 24 hour withdrawal, the MRLs recommended for fat (skin/fat where applicable) and liver are twice the LOQ of 25 µg/kg for the HPLC-UV method and the MRLs recommended for muscle and kidney are twice the LOQ of 7 µg/kg for the HPLC-UV method. Based on the limited residue data available for cattle, residues are similarly low in cattle and the recommended MRLs can be extended to cattle tissues.

The Committee recommended MRLs of 50 µg/kg for liver and fat and 15 µg/kg for muscle and kidney for chickens and pigs as narasin A. The Committee recommended the same MRLs, as temporary MRLs, for cattle.

The Estimated Daily Intake was not estimated because there were insufficient data points to calculate the median values for residues. Using the model diet and a marker:total ratio of 5%, the MRLs recommended above would result in an intake of 255 µg per person per day, which represents approximately 85% of the upper bound of the ADI.

Before re-evaluation of narasin with the aim of recommending permanent MRLs in tissues of cattle, the Committee would require a detailed description of a regulatory method, including its performance characteristics and validation data. This information is required by the end of 2010.

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TILMICOSIN

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Addendum to the monographs prepared by the 47th meeting of the Committee and published in the FAO Food and Nutrition Paper 41/9

BACKGROUND

The forty-seventh meeting of the Committee (FAO/WHO, 1998) reviewed tilmicosin and established an ADI of 0-40 µg/kg body weight (0-2400µg per day for a 60 kg person). The following MRLs (µg/kg) for cattle, sheep and pigs were recommended:

Species	Food commodity				
	Muscle	Liver	Kidney	Fat	Milk
Cattle	100	1000	300	100	
Sheep	100	1000	300	100	50 (T)
Pigs	100	1500	1000	100	

The temporary MRL of 50µg/kg for sheep milk was not extended by the Committee at the fifty-fourth meeting as results of a study with radioactively labeled drug in lactating sheep to establish the relationship between total residues and parent drug in milk was not available. The present addendum addresses both new and relevant previously submitted data.

The sponsor has requested MRLs for tilmicosin in chicken, turkey and rabbit tissues and chicken eggs in addition to a MRL for sheep milk. In this submission the sponsor explains the reasons for not having provided a total residue study in sheep milk using ^{14}C-tilmicosin as requested by the forty-seventh meeting of Committee.

IDENTITY

IUPAC Name: (5S,6S,7R,9R,11E,13E,15R,16R)-6-[(2R,3R,4S,5S,6R)-4-dimethylamino-3,5-dihydroxy-6-methyloxan-2-yl]oxy-7-[2-(3,5-dimethylpiperidin-1-yl)ethyl]-16-ethyl-4-hydroxy-15-[[(2R,3R,4R,5R,6R)-5-hydroxy-3,4-dimethoxy-6-methyloxan-2-yl]oxymethyl]-5,9,13-trimethyl-1-oxacyclohexadeca-11,13-diene-2,10-dione

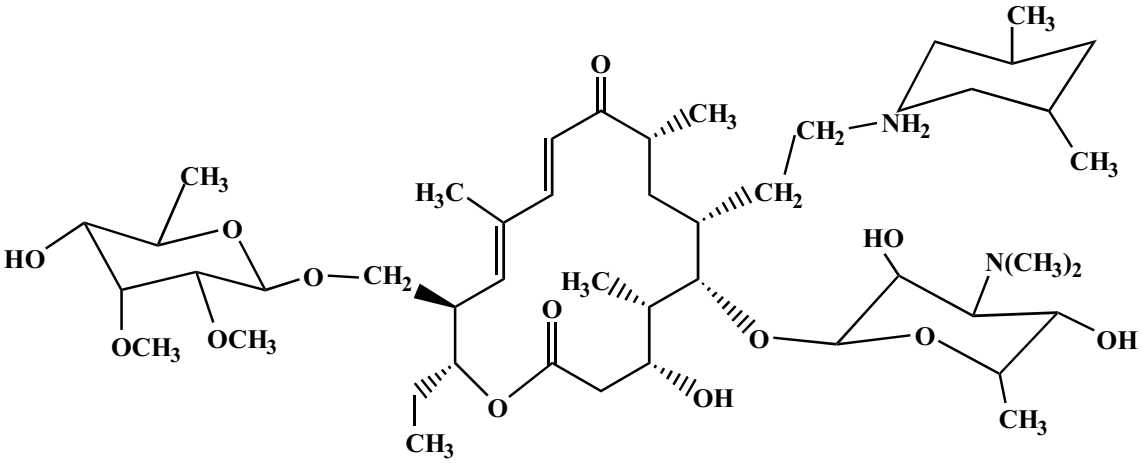
CAS Name: Tylosin,A-O-de(2,6-dideoxy-3-C-methyl-alpha-L-ribo-hexopyranosyl)-20-deoxy-20-(3,5-dimethyl-1-piperidinyl)-(20(cis: trans))

Other names: 20-dihydro-20-deoxy-20-(cis-3,5- dimethylpiperidin-l-yl)-desmycosin

CAS Number: 108050-54-0

Synonyms: NCBI PubChem Compound lists 19 synonyms
(Examples: Tilmicosin, Micotil, Micotil (TN), Micotil 300)

Structural formula:



Molecular formula: $C_{46}H_{80}N_2O_{13}$ (tilmicosin)
$C_{46}H_{83}N_2O_{17}P$ (tilmicosin phosphate)

Molecular weight: 869.133 [g/mol] (tilmicosin)
967.128 [g/mol] (tilmicosin phosphate)

OTHER INFORMATION ON IDENTITY AND PROPERTIES

Active ingredient: Tilmicosin is a white to off-white solid comprised of a *cis* isomer and a diastereomeric pair of *trans* isomers. The ratio of *cis* to *trans* isomers is about 85:15, respectively.

Melting point: Melting points of commercial products are not regularly given. 107-112°C and 143-149 °C can be found for certain commercial products of tilmicosin and tilmicosin phosphate, respectively.

Solubility: Tilmicosin base has a solubility of 1500 mg/L in n-hexane and solubility of up to >5000 mg/L in other organic solvents, e.g., acetone, acetonitrile, chloroform, dichloromethane, ethyl acetate, methanol, and tetrahydrofuran. Solubility in water and distribution between aqueous and organic phases is strongly pH-dependent (Xu, et al., 2006). The pKa values of tilmicosin *cis* and *trans* isomers are 7.4 and 8.5, respectively, in 66% dimethylformamide. At pH 9, the solubility is 7.7 mg/mL at 25°C and 72.5 mg/mL at 5°C. At pH 7 and 25°C, the solubility is 566 mg/mL.

Purity: Commercial products are of variable purity and isomeric composition (Stoev and Nazarov, 2008). Typical products may consist of 82-88 % *cis* isomer and 12-18% *trans* isomer

UV-absorbance: Tilmicosin exhibits a UV absorbance maximum at 284 nm. When in solution, tilmicosin is light sensitive.

RESIDUES IN FOOD AND THEIR EVALUATION

Condition of use

Tilmicosin is a macrolide antibiotic developed for veterinary use. The following are examples of recommended uses for the prevention and treatment of diseases caused by tilmicosin sensitive microorganisms:

- Pigs: treatment and prevention of pneumonia caused by *Actinobacillus pleuropneumoniae, Mycoplasma hyopneumoniae* or *Pasteurella multocida.*
- Cattle: Treatment and metaphylaxis of respiratory diseases caused by *Mannheimia haemolytica* und *Pasteurella multocida*. Tilmicosin is not to be used in cattle producing milk for human consumption.
- Sheep: For the treatment of pneumonia associated with *Mannheimia haemolytica* und *Pasteurella multocida*; for the treatment of ovine mastitis associated with *Staphylococcus aureus* and *Mycoplasma agalactiae* and as an aid in the control of enzootic abortion in ewes caused by *Chlamydia psittaci*.
- Rabbits: therapy of respiratory tract infections caused by *Pasteurella multocida* and *Bordetella bronchiseptica* and of bacterial enteritis caused by *Clostridia*.
- Chickens: For the treatment of respiratory infections in chicken flocks, associated with *Mycoplasma gallisepticum, M. synov*iae and other organisms sensitive to tilmicosin.
- Turkeys: For the treatment of respiratory infections in turkey flocks, associated with *Mycoplasma gallisepticum*. Tilmicosin is currently not to be used in chickens and turkeys producing eggs for human consumption.

Dosage

On the request of the Committee the sponsor provided copies of approved labels from a several countries. The information given in table 1 was extracted from the label instructions. Species not subject to a detailed review in the present monograph are given in squared brackets and no further details are included in the table. In summary: the currently recommended modes of administration include (examples only) subcutaneous injection in pigs, cattle and sheep, oral administration via feed in rabbits and pigs, and administration via drinking water in pigs, calves, chickens and turkeys and via milk, milk replacer in calves.

Table 1: Conditions of registered uses of tilmicosin in selected countries.

Country	Product	Target species	Treatment	Daily dose [mg/kg bw]	Withdraw time days]	Warnings and related texts
Austria	PULMOTIL ® Premix 20%, granulate	**Rabbit** [pigs]	Respiratory diseases: 100-200 ppm in feed, 7 days	10-12	5	
			Bacterial enteritis: 40-80 ppm in feed, 7 days	5-6		
Ireland	Pulmotil AC tilmicosin	**Chicken**	75 mg/L in drinking water, 3 days	10-25	12	Not to be used in chickens and turkeys producing eggs for human consumption.
		Turkey		6-30	15	
		[pigs]				
France	PULMOTIL AC Usage veterinaire Tilmicosine, Formulation aqueuse	**Chicken**		15-20	12	Not to be administered to hens producing eggs for human consumption
		Turkey		10-27	19	
Switzerland	Pulmotil AAC ad us.vet. liquid premix	**Chicken** [calves, pigs]	30-40 mL Pulmotil/100 mL of drinking water, 3 days	15-20	12	At the exception of laying hens producing eggs destined for consumption
Philippines	TILMICOSIN PHOSPHATE Pulmotil AC	**Chicken** [pigs]	75 mg/L in drinking water, 3-5 days for prevention, 5-7 days for treatment		10	Contraindication: Should not be used in birds producing eggs for human consumption.
Ireland	Micotil	**Sheep** [cattle]	Single dose of 10 mg/kg bw (1 ml Micotil /30kg)			Not for use in cattle producing milk for human consumption.

PHARMACOKINETICS AND METABOLISM

A number of studies provided information on pharmacokinetics, metabolism and on tissue residue depletion in target animal species. In such cases major pharmacokinetic findings are briefly summarized in this section and more details and data evaluations are given below in the section on tissue residue depletion studies.

Ruminants

A published study (Modric, et al. 1998) compared the pharmacokinetics of tilmicosin in cattle and sheep after subcutaneous administration of a dose of 10 mg/kg bw. The pharmacokinetic parameters derived from the time concentration curve (T_{max}, C_{max}, $T_{1/2}$, AUC) were not significantly different between species. Individual animal data were not provided and no information about equivalency of tissue distribution and metabolism could be derived.

A peer-reviewed pharmacokinetic study of tilmicosin in goats studied the bioavailability of tilmicosin after intravenous or subcutaneous administration of 10 mg/kg (Ramadan 1997). Concentrations in plasma and milk of goats were determined by a microbiological assay (LOD =5 ng/ml, LOQ = 10 ng/ml). A small fraction of tilmicosin was absorbed very slowly. C_{max} in plasma was 1.56 µg/ml. Tilmicosin was excreted in milk with a mean concentration peak of 11.6µg/mL and a slow depletion rate maintaining detectable concentrations more than 5 days after administration.

A GLP compliant radiometric study was performed in cows which were approximately two months from calving (Donoho and Thomson 1990). Radio-labeled tilmicosin was administered subcutaneously at a single dose of 10 mg/kg bw. The animals were managed as dry cows until parturition and milk samples collected after this time. In colostrums, tilmicosin represented 89 % of the total radioactive residue, which means that the administered dose remained largely unchanged for a long period since the interval between dosing and calving was around 50 days.

Chickens

Studies using ^{14}C-labelled tilmicosin

In a GLP compliant study (T5C749505, Ehrenfried, et al. 1996a) four week old Hubbard White Mountain Cross chickens were given *ad libitum* access to ^{14}C-tilmicosin (specific activity 0.278 µCi/mg) in medicated drinking water for five consecutive days. Two concentrations in water were tested (25 and 50 mg/L, respectively). Of the animals receiving the higher dose two groups were formed. The animals of the lower dose group and one of the higher dose groups were sacrificed 7 days after the end of treatment. The remaining group was sacrificed 10 days after the end of the treatment. Radioactivity was determined by liquid scintillation counting in liver, kidney, thigh and breast muscle, abdominal and skin fat, and bile. Although dosing was variable it is evident that the concentrations of residues in liver and kidney of individual animals were strictly proportional to the dose the individual animals had received. High concentrations of residues were also found in bile. The concentrations in muscle and fat were very low. Table 2 provides a summary of the results of the study.

Table 2: Summary of the results of study T5C749505.

Concentration in water [mg/L]	Animal	sex	Withdrawal time [days]	Dose [mg/kg]	Concentration in tissues [mg/kg]					
					Liver	Kidney	Breast muscle	Abdominal fat	Skin fat	Bile
25	9732	m	7	32.2	0.42	0.33	0.02	0.01	0.02	0.49
25	9739	m	7	20.7	0.21	0.14	<LOD	<LOD	<LOD	
25	9751	m	7	20.0	0.37	0.12	<LOD	<LOD	0.02	0.3
25	9708	f	7	21.0	0.14	0.14	<LOD	<LOD	<LOD	
25	9711	f	7	21.0	0.85	0.26	0.02	0.02	0.03	0.45
25	9715	f	7	19.0	0.15	0.16	<LOD	<LOD	<LOD	<LOD
50	9729	m	7	50.0	0.69	0.41	<LOD	0.02	0.06	0.91
50	9731	m	7	48.2	0.6	0.33	<LOD	0.02	0.05	0.69
50	9730	m	7	50.7	0.72	0.39	0.03	0.04	0.07	0.79
50	9717	f	7	51.3	0.4	0.31	<LOD	<LOD	0.02	0.25
50	9721	f	7	33.1	0.47	0.44	0.02	0.01	0.03	0.22
50	9709	f	7	43.2	0.85	0.37	0.02	0.02	0.03	
50	9742	m	10	76.9	1.96	0.7	0.03	0.02	0.07	
50	9749	m	10	52.5	1.04	0.47	<LOD	0.02	0.05	0.9
50	9738	m	10	44.5	0.8	0.36	0.04	0.02	0.05	0.59
50	9716	f	10	43.9	0.33	0.21	<LOD	<LOD	0.03	0.16
50	9720	f	10	34.5	0.3	0.18	<LOD	0.01	<LOD	0.12
50	9718	f	10	44.8	0.24	0.16	<LOD	<LOD	0.02	

LODs were given in cpm and based on the lowest count which was significantly above the background.

In another GLP compliant study (T5C749601, Ehrenfried, et al. 1996b) two groups of four weeks old Cornish Cross chicken were treated with ^{14}C-tilmicosin (specific activity 2.87 µCi/mg) for five consecutive days followed by a seven day withdrawal period. In the first group six birds were given ad libitum drinking water containing 100 mg/l of ^{14}C-tilmicosin; in the second group four birds were dosed by oral gavage twice daily at 11 mg/kg bw/day. There was some variability in the dosing via drinking water and females consumed significantly lower amounts of medicated water than males. Following sacrifice radioactivity was determined by liquid scintillation counting in liver, kidney, thigh and breast muscle, abdominal and skin fat. The results are summarised in table 3.

In another GLP compliant study (T5C749504, Ehrenfried, et al. 1997a) three groups of four week old Cornish cross chicken were dosed *ad libitum* with ^{14}C-tilmicosin (specific activity 3.13 µCi/mg) for five consecutive days. The concentrations of tilmicosin in drinking water were 150, 300, and 450 mg/l, respectively. The first and third group were sacrificed six hours after their last exposure to medicated water. Group 2 was sacrificed after 5 days withdrawal time. The entire liver minus the gall bladder, both kidneys, thigh and breast muscle, abdominal fat, skin with attached subcutaneous fat (skin fat), the brain, both lungs, bile and excreta were collected and analysed. The results are summarised in table 4.

Table 3: Results of the tissue analyses of study T5C749601.

Animal	Sex	Average daily dose [mg/kg]	Withdrawal time [days]	Concentrations in tissues [mg/kg]					
				Liver	Kidney	Muscle	Abdominal Fat	Skin Fat	Bile
6564	m	23.2	7	5.24	1.91	0.22	0.08		
6559	m	34	7	5.13	2.01	0.2	0.12		6.6
6565	m	23.5	7	2.8	1.3	0.07	0.05		2.3
6573	f	15.2	7	4.53	1.3	0.18	0.08	0.24	1.9
6576	f	17	7	4.05	1.6	0.13	0.07	0.22	4.6
6574	f	18.2	7	2.41	1.19	0.06	0.05	0.11	1.4
6557	m	22	7	4.37	2.18	0.12	0.12	0.29	7
6561	m	22	7	2.96	2.07	0.08	0.07	0.26	2.8
6572	f	22	7	2.75	1.44	0.08	0.06	0.1	2.6
6571	f	22	7	3.75	1.57	0.1	0.07	0.19	3.1

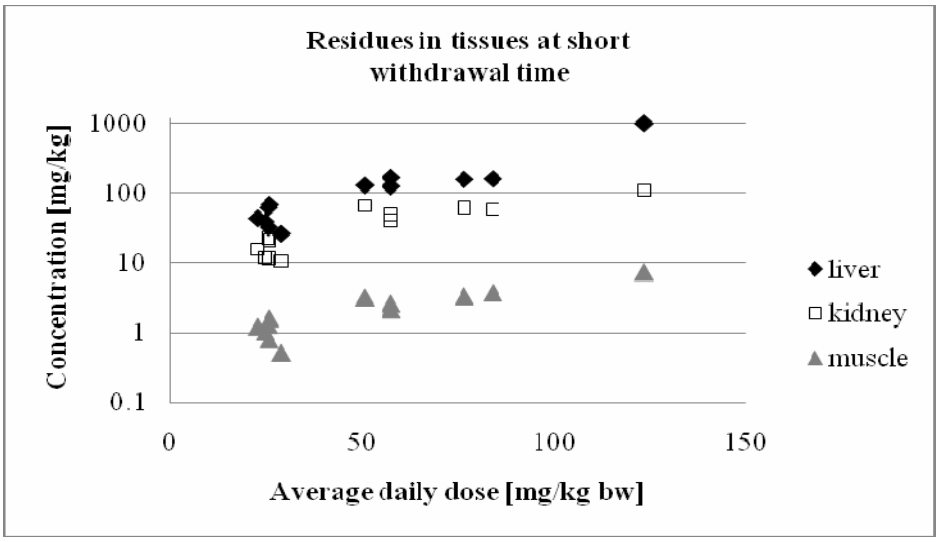
Table 4: Results of the tissue analyses carried out in study T5C749504.

Animal	Sex	Average daily dose [mg/kg bw]	Withdrawal time [days]	Liver	Kidney	Muscle	Abdominal Fat	Skin Fat	Brain	Lung	Bile
				Concentration [mg/kg]							
6531	m	29.0	0.25	25.9	10.6	0.5	0.4	0.6	0.2	2.0	71.4
6538	m	26.0	0.25	68.1	20.9	1.6	1.0	1.5	0.4	5.8	385
6542	m	25.8	0.25	32.0	11.6	0.8	0.6	0.9	0.2	2.6	212
6501	f	25.7	0.25	61.5	22.5	1.3	1.0	1.4	0.3	6.4	216
6504	f	22.8	0.25	43.1	15.6	1.2	0.7	1.3	0.4	5.8	122
6506	f	24.9	0.25	38.8	11.7	1.0	0.7	1.0	0.2	3.2	167
6528	m	50.2	5	73.1	13.4	0.8	0.6	1.4	1.1	9.2	47.6
6543	m	50.7	5	19.6	4.6	0.4	0.3	0.8	0.5	2.7	22.1
6546	m	52.9	5	16.8	6.0	0.3	0.4	0.7	0.3	1.7	13
6505	f	46.0	5	16.6	5	0.4	0.3	0.6	0.4	2.3	14.9
6523	f	51.8	5	5.9	3.3	0.2	0.2	0.3	0.1	1.2	8.4
6527	f	46.4	5	10.6	4.4	0.2	0.2	0.3	0.3	1.2	8.5
6532	m	76.7	0.25	157	62.3	3.4	2.8	3.8	0.7	21.4	1456
6533	m	123.5	0.25	1007	109	7.5	4.7	6.0	1.4	33.0	10650
6548	m	84.2	0.25	160	59.1	3.8	2.6	3.8	0.5	10.3	840
6507	f	50.8	0.25	129	65.5	3.2	3.5	3.0	0.8	15.0	809
6510	f	57.6	0.25	125	40.3	2.6	2.0	2.7	0.6	11.4	802
6518	f	57.6	0.25	168	49.2	2.2	1.2	2.5	0.6	10.1	603

It is evident that the intended high dose could not be achieved in females and the results were highly variable in males. Liver was the edible tissue with the highest concentration of ^{14}C-tilmicosin-equivalents. The concentrations of radioactive residues were very high in samples of bile collected early after the end of the treatment of the animals. At later time points they were in the order of the concentrations found in liver. The concentrations of residues determined in tissues of animals of groups one and three can be directly compared because the withdrawal time was the same (6 hours). These results are plotted in figure 1 as function of the determined average daily dose. The curves for the three tissues are approximately parallel. Reviewing each tissue individually using the most appropriate linear scaling (not shown) there is strict proportionality between the achieved dose and the

concentration of residue – with the exception of one outlying point for liver in the animal that had received the highest dose. Dose linearity is also clearly seen in other studies.

Figure 1: Initial concentrations of total residues in edible tissues as function of dose.



The authors found that approximately 70% of the administered doses were excreted by the end of the treatment period and that excretion had probably reached a steady state at that time. Figure 2 shows the concentrations of radioactive residues in excreta collected on every treatment day. The results seem to confirm this statement despite some variability observed in the highest dose group which might be explained on the basis of the variability of the doses achieved. This is illustrated in figure 3 where the concentrations in excreta in males and females observed on the last treatment day are plotted as function of the average daily dose.

Figure 2: Concentration of radioactive residues (tilmicosin equivalents) in excreta.

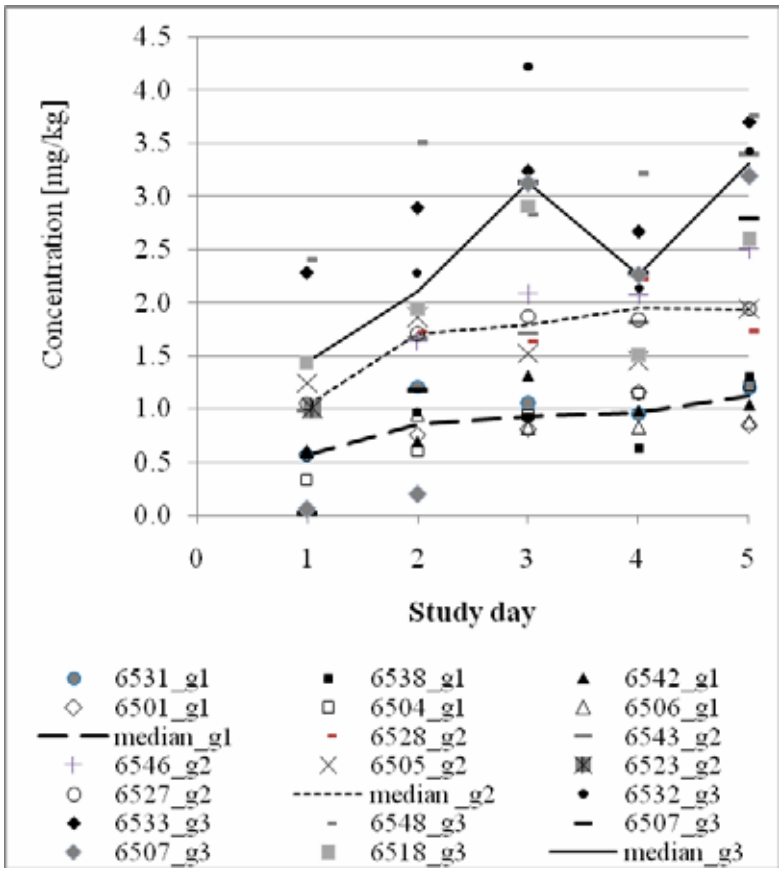
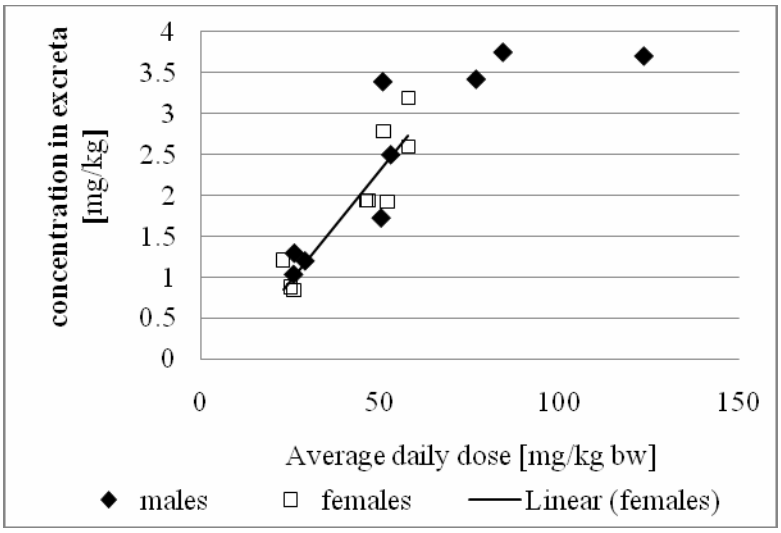


Figure 3: Day 5 concentration of radioactive residues [tilmicosin equivalents] in excreta.



Extracts of liver, kidney, muscle, lung, excreta and bile were prepared and the extracts were subjected to cleanup and complex partitioning schemes. The fractions were analysed by HPLC and radioactivity was determined. The structure of metabolites was determined using ESP-MS. In total, a number of metabolites and parent tilmicosin were found in the extracts. The structures are briefly described in table 5.

Table 5: Main metabolites found in tissues and excreta of chicken in study T5C749504.

Compound	Description
Parent tilmicosin	Including tilmicosin cis-8-epimer
T-1	Tilmicosin desmethylated at the dimethylamine portion of the mycaminose ring
Oxitilmicosin	A form of tilmicosin epoxidised at the macrolide ring
T-3	Replacement of the dimethylamine portion of the mycaminose ring with a hydroxyl group
T-4	Reduced form of tilmicosin, sulphated at the C11 position
T- 6	Tilmicosin devoid of the dimethylamine portion of the mycaminose ring
T- 7	Dehydroxylated form of tilmicosin devoid of the dimethylamine portion of the mycaminose ring
T- 8	Tilmicosin methylated at the mycaminose substituent
T-9	Tilmicosin devoid of its mycinose moiety
T-10	Metabolite T-1 devoid of mycinose moiety

Table 6 summarises the percent of total radioactivity attributable to the parent and major metabolites. All values are expressed in % of total radioactivity. The results suggest that in liver approximately 55% of the total radioactive residue represents parent tilmicosin. The corresponding values for kidney and muscle are approximately 40%.

Table 6: Metabolite profiles of tissues and excreta in chicken of study T5C749504.

Tissues and metabolites	Treatment groups					
	1		2		3	
	females	males	females	males	females	males
Liver	% of total radioactive residue					
Tilmicosin	49.6	55.3	36	50.2	62.3	67.7
T-1	6.6	4.8	5.3	9.1	5.4	4.7
T-2	2	1.7	2.2	4.7	2.2	1.8
Traces T-6, T-7						
Kidney						
Tilmicosin	52.2	36.1	25.2	34	49.1	43.3
T-1	7.1	7	4.9	5.2	9	7.5
T-2	1.7	1.4	1.3	1.2	1.6	1.6
T-9	4.8	2	19.9	12	3.2	2.1
T-10	2.4	1.5	6.7	3.2	1.5	1.1
Muscle						
Tilmicosin	41.8	50.8	25.4	28.8	47.1	37.2
T-1	8.7	6.2	7.4	12.3	12.8	27.5
Lung						
Tilmicosin	37.5	32.5	13	31.4	43.7	53.3
T-1 plus T-3	18.1	21.5	10.3	11.3	14.2	13
Bile						
Tilmicosin	80.9	NA	57	70.7	84.1	83
T-1	3.9	NA	NQ	7.9	3.5	3.9
T-4	2.3	NA	NQ	NQ	2.4	1.3
Oxy-tilmicosin	2.3	NA	NQ	4.8	2.6	3.8
Excreta						
Tilmicosin	31.2	41.9	33.2	36.8	31.5	30.7
T-1	7.4	7.8	5.9	7.2	9.5	8.7
T-4	35.7	26.4	37.7	33	33.1	39.2
Traces T-6, T-7, T-8						

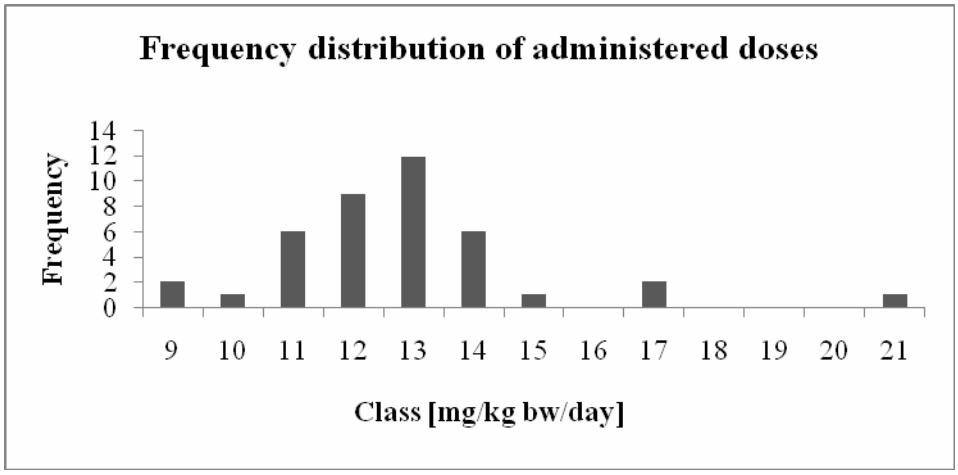
In another GLP-compliant study with ^{14}C-tilmicosin in chicken (T5C749602; Ehrenfried, et al, 1997b), five groups of eight (4 of each sex) 4-week old Cornish Cross chickens received [^{14}C]-tilmicosin (specific activity 2.62 µCi/mg) in medicated drinking water at concentration of 75 mg/L ad libitum for three consecutive days. At withdrawal times of 3, 7, 10, 14, and 21 days one group was sacrificed. The entire liver minus the gall bladder, both kidneys, samples of thigh and breast muscle, abdominal fat, skin fat, bile and excreta were collected from each animal and analysed for total radioactivity by liquid scintillation counting following solubilisation. Four randomly selected samples of liver and kidney from each group and four randomly selected muscle samples from the animals sacrificed 3 and 7 days after the end of treatment were also analysed for parent tilmicosin.

Body weights of the birds were determined twice, the first time before the dosing and the second time after dosing. The authors used the average; this is justified because the weight gains during the dosing period were up to 350g per bird. The achieved doses were variable ranging from 8.5 to 20.4mg/kg bw/day (average 12.3 ± 2.1mg/kg bw/day]. Doses were slightly higher and slightly more variable in males than in females. A frequency distribution of the doses is given below in figure 4.

The highest residue concentrations were observed in liver followed by kidney. Residue concentrations in skin fat, abdominal fat and muscle were very low. The variability of the data was high. A few data points exhibited extreme values. However, no data points were excluded from statistical analysis. A

kinetic analysis based on linear regression was performed. The results are discussed in the below section on tissue residue depletion studies.

Figure 4: Frequency distribution of doses achieved in study T5C749602.



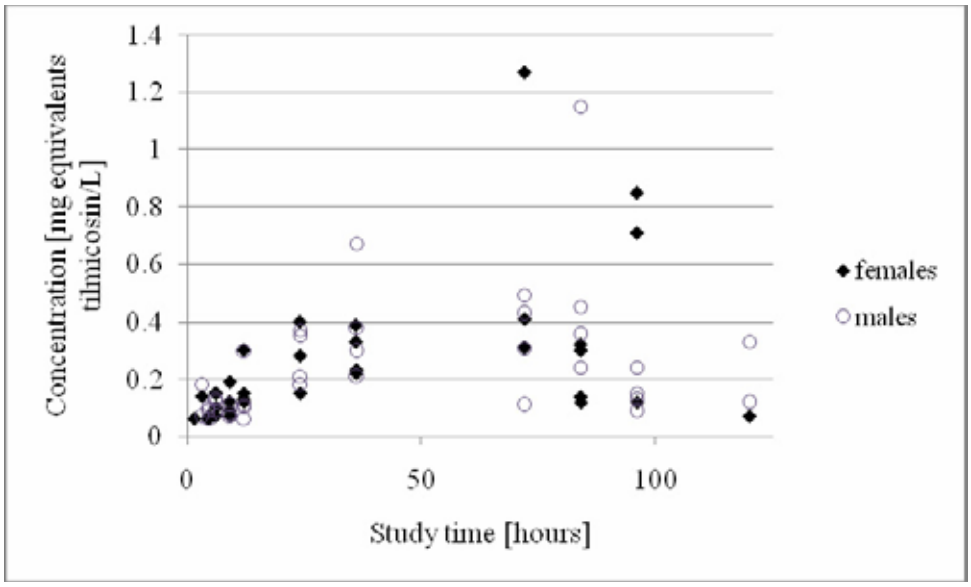
Another GLP compliant study (96 – ELA – 01, Peters, et al., 1997) involved 184 broiler chickens (92 of each sex). At an age of 3 – 4 weeks groups of animals were treated using three concentrations of tilmicosin in drinking water. Water was provided *ad libitum* from hanging drinkers. Body weight ranged from 348 to 758 g per animal on day -2. There were no significant differences between body weights of the groups. Time zero of treatment was staggered between groups in order to allow scheduling of blood samples. Achieved doses were calculated on the basis of group water intakes. Table 7 summarises the results of dosing. The grand average of the administered doses was 12.8, 21.8, and 56.0 mg/kg bw/day for the low dose, middle dose and high dose group respectively.

Table 7: Doses achieved in study 96 – ELA – 01.

Study day	Targeted concentrations in drinking water [mg/L]	Measured concentrations in drinking water [mg/L]	Achieved doses [mg/kg bw/day]		
			Minimum	Maximum	Average
0	37.5	34	10.3	13.3	11.7
	75	67	19.6	23.6	21.6
	150	138	40.3	54.4	46
1	37.5	36	10.5	19.1	13
	75	68	20.1	23.1	21.6
	150	210	61.2	75.2	68.8
2	37.5	34	10.4	23.7	13.8
	75	66	19.6	24.9	22.3
	150	137	45.6	65.9	53.2

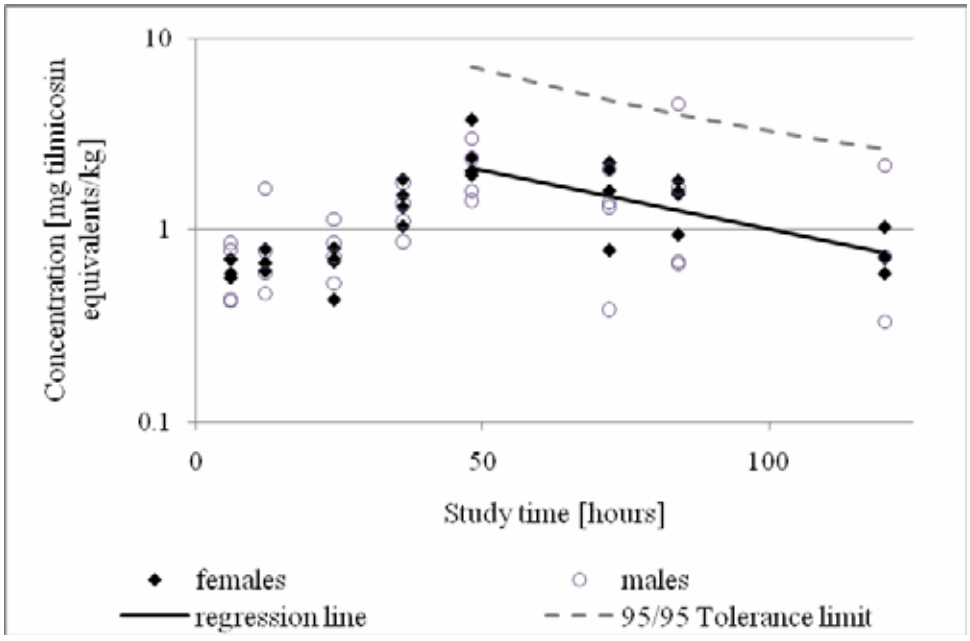
Serial blood samples were taken from the wing veins of eight birds of each group from time 0 to 120 hours after treatment. If both wing veins collapsed of developed hematomas spare chickens were used. Some haemolysed samples could not be used for the analyses. In addition, samples with a volume below 0.8 ml could not be analysed. Analyses were performed in plasma using a validated HPLC method and UV detection. Only for the high dose group there were sufficient measured values to produce a graph. The results are shown in figure 5. Results were variable and there were no significant differences observed between males and females. T_{max} cannot precisely determined because there was a data gap between 36 and 72 hours and the results obtained at 72, 84, and 96 hours were highly variable.

Figure 5: Radioactive residues in plasma samples obtained in study 96 – ELA – 01.



Four male and four female birds of the middle dose group were slaughtered at several time points from 6 to 120 hours after begin of treatment and lungs and airsac tissues were analysed using a validated HPLC method with UV detection. Since only small amounts of airsac tissue could be obtained from the animals all tissues sampled at a given time point were pooled and analysed as one sample. The total radioactive residue increased from the beginning of treatment until approximately hour 48 of the study. The depletion of the residues could be described by linear regression on a semi-logarithmic scale. Compared to plasma the residues accumulated in lungs. The results are given in figure 6.

Figure 6: Kinetics of formation and depletion of total radioactive residues in lung tissues obtained in study 96 – ELA – 01.



High concentrations of total residue also accumulated in airsac tissues. The analytical results obtained with pooled tissues of animals treated with the middle dose are shown in table 8.

Table 8: Residues in airsac tissues.

Study time [hours]	Concentration [mg equivalents tilmicosin/kg]
6	0.3
12	0.52
24	0.89
36	1.79
48	3.29
72	2.38
84	3.1
120	2.86

Turkeys

A study with unlabeled tilmicosin was performed to identify metabolites in turkey liver using HPLC-ESP-MS (Study 870 566, Ehrenfried et al. 1998). Parent drug was the main component of the extract, supporting it as marker residue for turkey.

Laying hens/eggs

Eight laying hens received by gavage, two times by day, a dose close to 10 mg/kg bw of ^{14}C-tilmicosin, during three days (SBL 004-00780, Beauchemin, et al. 2007a). Total radioactivity was determined in egg white and yolk during 24 days after the beginning of treatment. Pools of egg whites and egg yolks were extracted and analysed by HPLC-MS/MS to determine the metabolites. The ratio of tilmicosin to total residue was calculated and a value of 0.7 was estimated from the data base provided.

TISSUE RESIDUE DEPLETION STUDIES

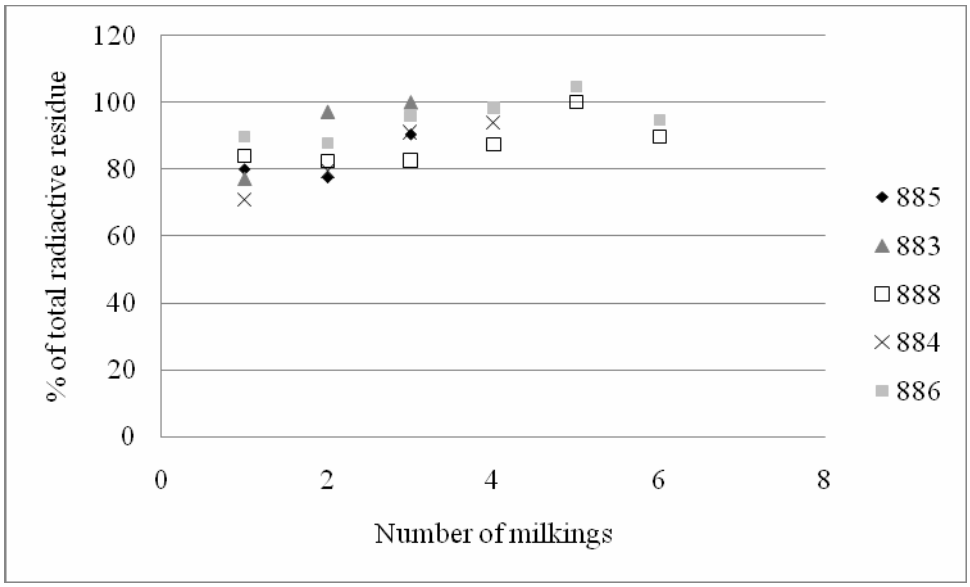
Studies in milk producing animals

Cattle

Study with ^{14}C-labelled tilmicosin

Five Holstein cows which were approximately two months from calving were injected subcutaneously with ^{14}C-tilmicosin of a specific activity of 1.28 µCi/mg at a single dose of 10 mg/kg bw (Study ABC-0447, Donoho and Thomson 1990). The study was GLP compliant. The animals were managed as dry cows until parturition. Milk samples were collected twice daily after this time and assayed for total radioactivity by liquid scintillation counting. The milkings analysed were numbers 11, 27, 14, 15, and 19, respectively for the five animals in the study. The first three to six milkings were also analysed for tilmicosin following extraction and fractionation on an HPLC column. The first milkings are usually considered colostrum unfit for human consumption. The seventh milking represents about the first which could be marketed for human consumption. With one exception no milking suitable for human consumption was analyzed. In this exceptional case it was the 15th milking obtained from one cow and the concentration of residues was below the limit of detection. In the other colostrum samples the parent drug tilmicosin represented 88.9 ± 8.8% of the total radioactive residue. This is an important finding since the interval between dosing and calving was 52, 50, 44, 59, and 49 days respectively. During this long time the administered dose remained largely unchanged in the bodies of the animals and there was no significant time trend observable over the first six milkings (see figure 7).

Figure 7: Percent of parent drug tilmicosin in the total radioactive residue in cow's milk.

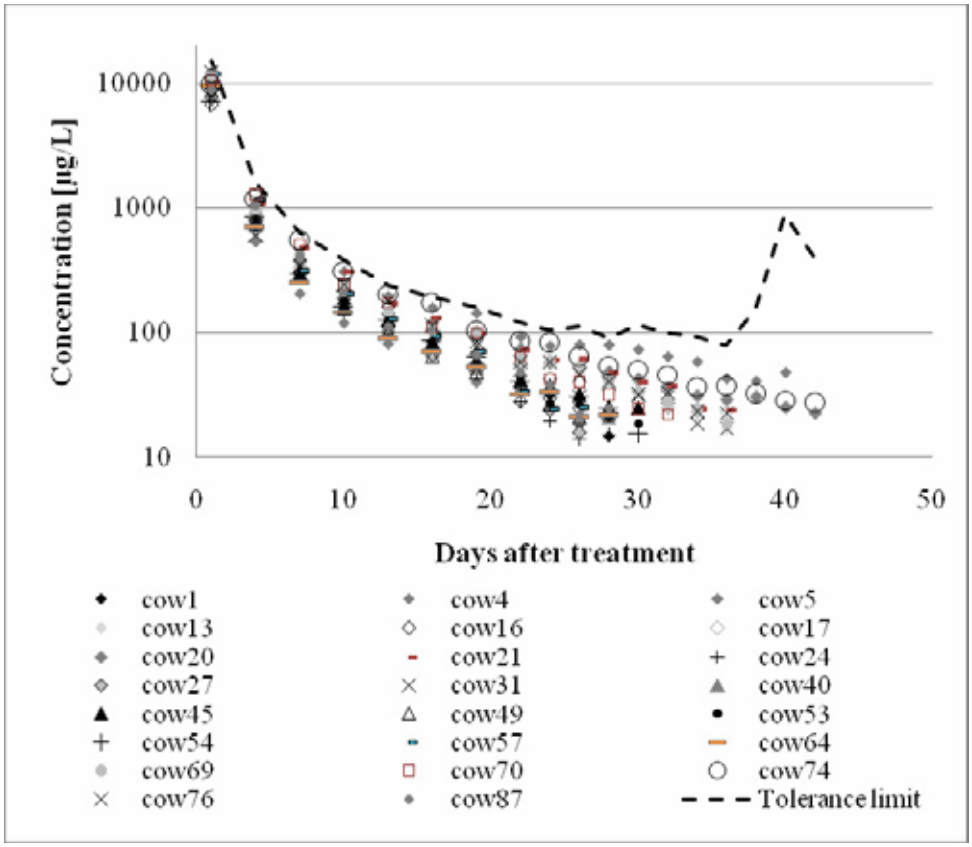


Studies with unlabelled tilmicosin

The depletion of tilmicosin was also investigated in a GLP compliant study with Holstein dairy cows (A03586/T5CCFF0301, Lacoste 2003). 25 animals received a subcutaneous injection of Micotil 300® corresponding to 10 mg/kg (range from 10.1 to 10.4 mg/kg). The labels of registered products provided by the sponsors warn that tilmicosin should not be used in cows producing milk for human consumption.

Animals in early, mid, and late lactation were used. Milk samples were taken before treatment and every evening on days 1, 4, 7, 10, 13, 16, 19, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, and 42. The samples were analyzed using HPLC. Method validation details are not given. When two consecutive concentration values fell below 50 µg/kg, subsequent samples were not analysed. Therefore, the upper one sided confidence limit over the 95th percentile increases again after 36 days (see figure 8). If one would consider recommending an MRL on the basis of a 36 day milk discard time the value would be approximately 80 µg/kg, a concentration still causing inhibitory activity in the Delvotest.

Figure 8: Depletion of tilmicosin in cow's milk.

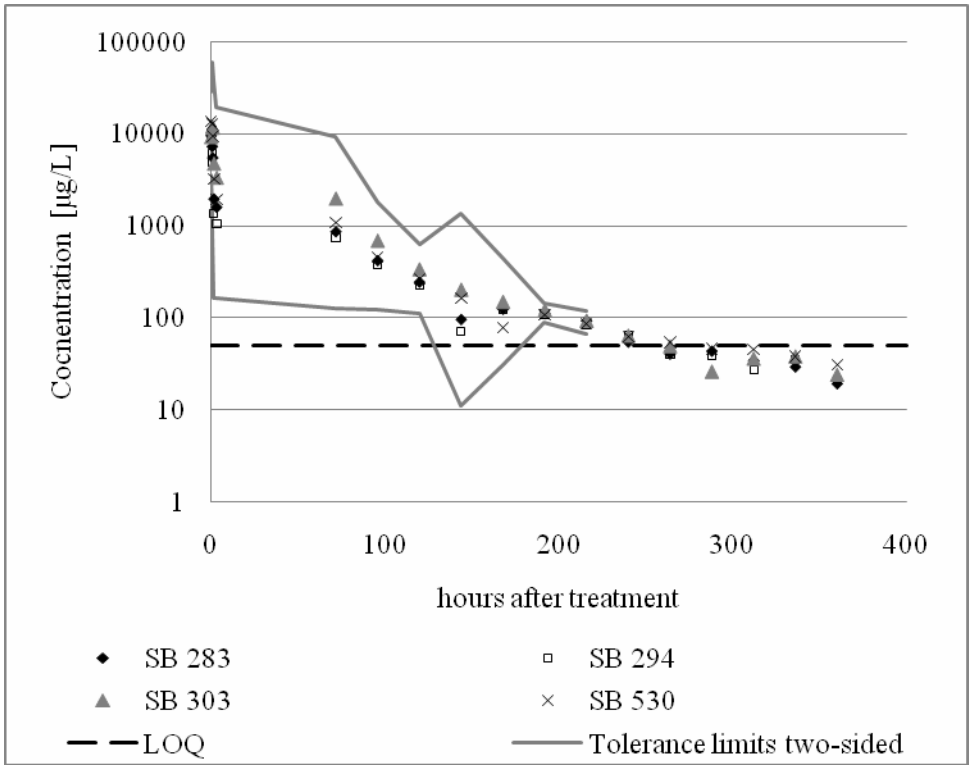


Sheep

A single subcutaneous dose of 10 mg/kg bw was given to 4 lactating Suffolk X ewes (CVLS3/92, Parker et al. 1992). They were all about 52 days after lambing and the lambs had been weaned seven days before the beginning of the study. Milk samples were taken from all four animals until day 28 after treatment. Milk was analysed for parent tilmicosin using an HPLC method. It is stated in the report that the method had been validated and that the limit of quantification was 50 µg/l. The milk was also subjected to a Delvotest and full inhibition was found for the first 6 to 7 days. No inhibition in any sample was found after day 12. The range of concentrations of parent tilmicosin was from 26 to 46 µg/kg on this day. A number of samples contained residues at concentrations below the LOQ. In order to identify those samples a line corresponding to the LOQ is drawn parallel to the x-axe in figure 9.

The data base of this study is very limited. Figure 9 visualises the extreme distances (n=4) between the measured values and the calculated (here a 2-sided for better visualisation) tolerance limits. These limits cannot be derived from linear regression like in the case of edible tissues of poultry and slaughter animals because the data points on the depletion curves are obtained from the same four animals every day. The weaknesses of the study cannot be compensated by recommending high MRLs. Consumption of milk obtained within the first 144 hours after treatment likely leads to intakes exceeding the ADI.

Figure 9: Depletion of tilmicosin in sheep's milk.



To consider recommending MRLs on the basis of longer milk discard times calculations like those shown in table 9 could be used. The MRL is derived from upper one-sided tolerance limits calculated in a conservative manner using the logarithms of the concentrations and calculating the antilog of the mean of the logarithms plus 6.37 standard deviations (for n=4).

Table 9: Example of the way of calculating MRLs for milk.

Withdrawal time [hours]	Mean (logarithms)	s.d. (logarithms)	k	One-sided Tolerance limit (antilog) [µg/l]	Intake equivalent to tolerance limit [µg/person/day]
168	2.06354	0.117100	6.37	645	1075
192	2.04976	0.022098	6.37	155	258
216	1.94515	0.025737	6.37	129	214

Another important consideration is that concentrations above 50 µg/l will most likely result in antimicrobial activity of the milk if tested in the Delvotest. While it seems possible to find MRLs in a way that the human gut flora is not affected, it would not be so to derive an MRL and a corresponding milk discard time from the available data base that provides insurance that the milk has no inhibitory properties. An MRL of 50 µg/l would require a milk discard time > 360 hour hours.

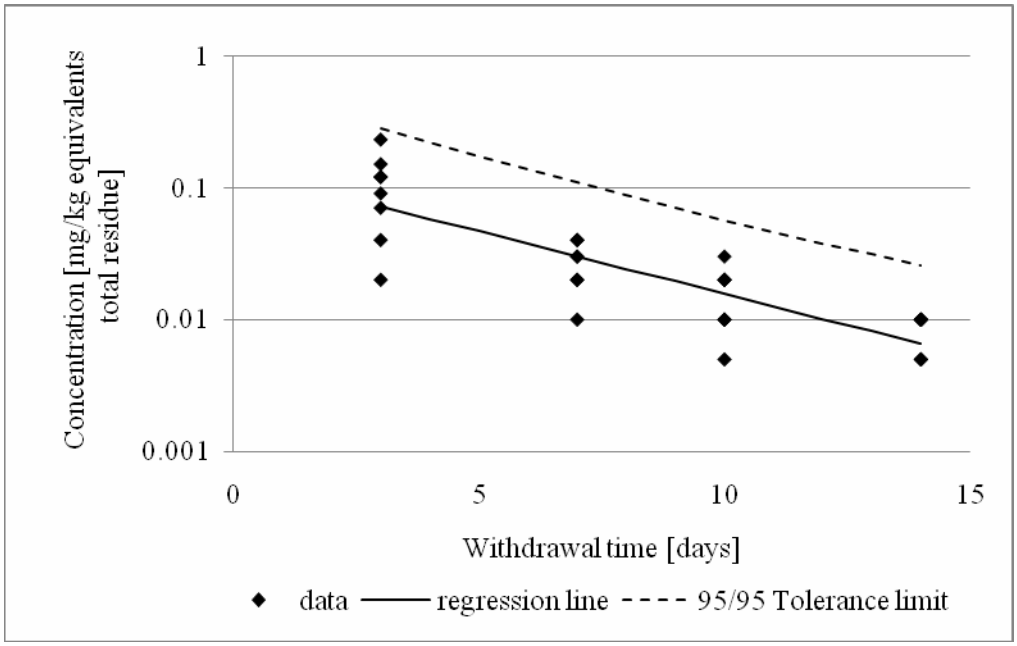
Chickens

Study with ^{14}C-labelled tilmicosin

A kinetic analysis based on linear regression was performed using the data of the above mentioned study T5C749602 (Ehrenfried, et al, 1997b). For liver and kidney all data points were used as given. For abdominal and skin fat the analysis was limited to 3-14 days withdrawal time because at later time

points too many concentrations were below the limit of detection. For non-detects occurring before or at 14 days, 0.005 mg/kg was substituted. For muscle only the data obtained for days 3-10 were suitable for statistical analysis. Non-detects were replaced by 0.01 mg/kg. Figure 10 gives an example of such analyses.

Figure 10: Example of statistical analysis of depletion data for skin fat.



The results of the statistical analysis are presented below in table 10. The authors have calculated averages of the results obtained on a given day for all animals. Since the data are not normally distributed and on day 3 there was one animal with extreme concentrations of residues in its tissues, such calculations can be misleading and suggest much higher residues than were encountered. The values predicted from the regression line and the calculated tolerance limits provide much more reliable estimates of the trends and the variability of the residue concentrations. Therefore it was preferred to perform such analysis even in cases where the data were only marginally suitable for this type of analysis. The results of this study are best suited to calculate the estimated daily intake (EDI) for total residues for the first ten days after treatment. For this time period results for all tissues are available. Skin fat was used in the food diet because of its higher concentrations of residues.

Table 10: Results of the statistical evaluation of kinetic residue data obtained in study T5C749602.

day	Liver		Kidney		Muscle		Skin fat		Abdominal fat	
	Predicted from regression line	Tolerance limit	Predicted from regression line	Tolerance limit	Predicted from regression line	Tolerance limit	Predicted from regression line	Tolerance limit	Predicted from regression line	Tolerance limit
	Concentration of total residue [mg/kg tilmicosin equivalents]									
3	2.94	20.18	0.88	3.03	0.11	0.71	0.14	0.45	0.07	0.28
7	1.56	9.93	0.66	2.18	0.04	0.24	0.07	0.20	0.03	0.11
10	0.97	6.01	0.54	1.74	0.02	0.12	0.04	0.11	0.02	0.06

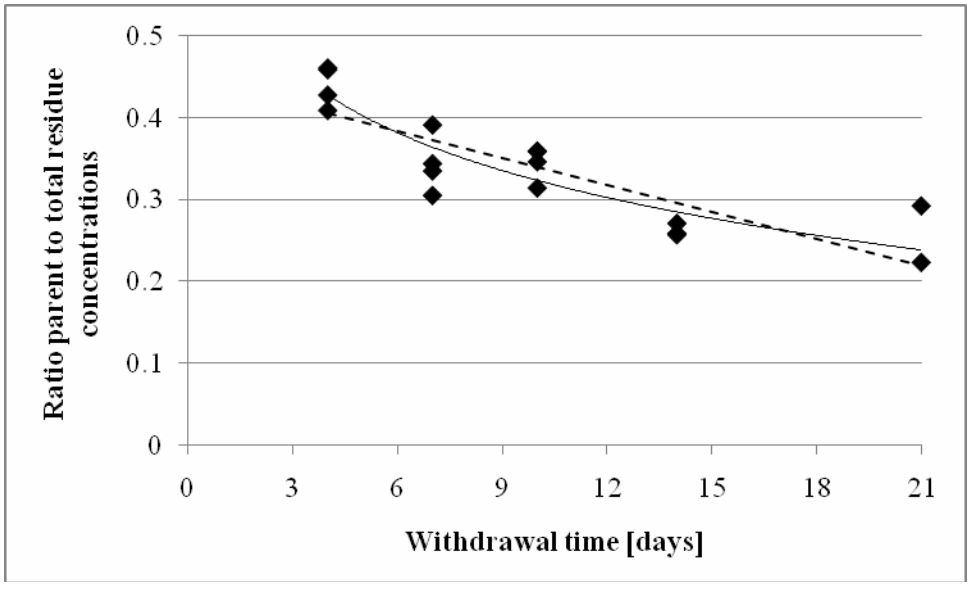
These values may need to be adjusted depending on the dose resulting from authorised treatments according to the label instructions. The information on the Irish label suggests a range of daily doses from 10 to 25 mg/kg of body weight. The French and Swiss labels assume a range of 15 to 20 mg/kg bw per day. In the "cold residue study" discussed below average daily doses ranged were 15. 9 – 20.9 mg/kg bw in females and 16.5 – 21.7 mg/kg bw in males. The average used in the study was 17.5 ± 2.2 mg/kg bw. The data given in table 11 are based on the unchanged results of the study. The ADI is 2400 µg/60 kg person/day.

Table 11: Calculation of the EDI of total tilmicosin related residue using data of study T5C749602.

day	Liver	Kidney	Muscle	Skin fat	Abdominal fat	All tissues	% of ADI
	EDI [µg/60 kg person/tissue/day]						
3	294	44	33	7.0	3.6	378	15.7
4	251	41	26	5.8	2.9	323	13.5
5	214	38	20	4.8	2.3	277	11.6
6	183	36	16	4.0	1.9	238	9.9
7	156	33	12	3.3	1.5	205	8.5
8	133	31	10	2.7	1.2	177	7.4
9	114	29	7	2.3	1.0	152	6.4
10	97	27	6	1.9	0.8	132	5.5

For a number of animals the concentration of parent drug was determined separately. For liver it was possible to establish a time trend which is given graphically in figure 11. The graph shows the data points and two possible trend lines (linear and logarithmic interpolation of the data). Similar time trends could not be established for other tissues. The ratio in kidney on day three was 0.3. The ratio in muscle did not change between days three and seven and was approximately 0.66.

Figure 11: Ratio of marker to total residue concentrations in liver.



Some representative tissue samples were extracted and metabolite profiles were determined. Table 12 shows percent of parent drug in tissue extracts. These values overestimate the parent to total ratio because the reference is the radioactivity in the extract and not the total radioactivity. They are used here to demonstrate that for kidney the ratio decreases over time. Because of the uncertainties in the determination of ratios it might be more appropriate to derive MRLs from a marker residue study and

calculate in parallel the corresponding intakes for each time point directly from the study discussed here, rather than to use highly uncertain conversion factors. The EDI would then represent a conservative "worst case" estimate.

Table 12: Percent of total extracted residue representing parent drug.

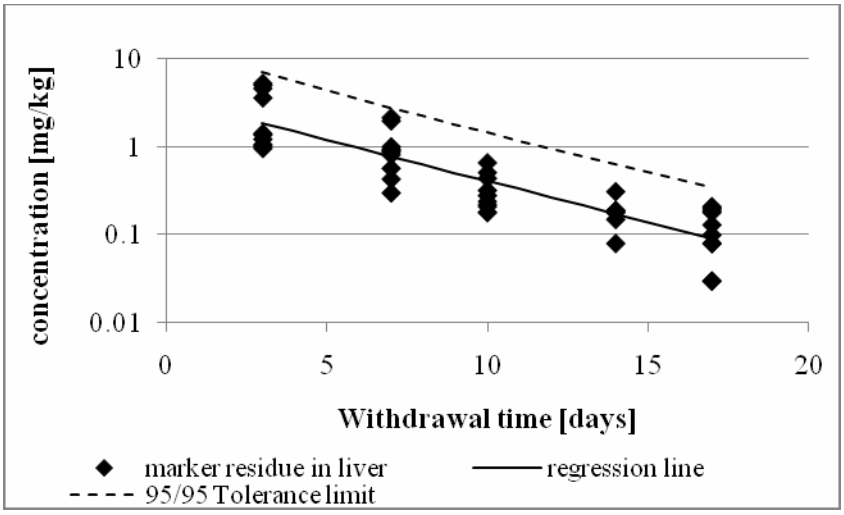
day	% parent tilmicosin in extract	
	Liver	Kidney
3	49.5	19.2
7	46.5	6.3
10	37.6	11.1
14	26.2	2.7
21	18.9	7.2

Studies with unlabelled tilmicosin

Chickens were dosed with tilmicosin in drinking water (75 mg/l) for three consecutive days in a GLP compliant study (T5C619610, Readnour, et al., 1997). Access to water was *ad libitum*. Five male and five female chickens were sacrificed on days 3, 7, 10, 17, and 21 after the end of treatment. Four males and three females were sacrificed 14 days after treatment. Three animals of this group were lost due to death or injury. Liver, kidney, breast and leg muscle, skin fat and abdominal fat were analysed. The limit of quantification was 0.06 mg/kg for liver and kidney (0.3 for day 17 and day 21 tissues) and 0.025 for muscle and fat.

The range of body weights of the animals was 890 – 1256g (mean 1065g) for males and 853 – 1170g (mean 976g) for females before the animals were treated. The report of the study does not provide individual animal based dosing information. The dose calculation was based on mean pen weight of the animals (for each animal the average of the body weights before and after treatment was used) and on total pen water intake. Even under these conditions of calculation average daily doses ranged from 15.9 – 20.9 mg/kg bw in females to 16.5 – 21.7 mg/kg bw in males. The average used in the study was 17.5 ± 2.2 mg/kg bw.

Figure 12: Depletion of marker residue in chicken liver.



The results of the determination of residues were subjected to statistical data treatment in this monograph. For liver it was possible to use all data points from 3 – 17 days withdrawal time. In kidney, too many results were below the LOQ after 10 days. For skin fat and muscle only the data for days 3 and 7 could be used. Results marked as below the limit of quantification were replaced by half

the LOQ. Figure 12 shows as an example the depletion of marker residue in liver. Table 13 summarises all results obtained by using statistical methods. Despite the limited number of data for some kinetics the statistical approach was considered the most appropriate to obtain quantitative information on both trends and variability.

Table 13: Results of the statistical evaluation of the chicken marker residue study.

day	Liver		Kidney		Muscle		Skin Fat	
	predicted from regression	Tolerance limit	predicted from regression	Tolerance limit	predicted from regression	Tolerance limit	predicted from regression	Tolerance limit
	Concentration [mg/kg of marker residue]							
3	1.83	7.12	0.54	2.54	0.08	0.49	0.10	0.47
7	0.77	2.82	0.14	0.61	0.03	0.16	0.05	0.24
10	0.40	1.45	0.05	0.24				
14	0.17	0.63						
17	0.09	0.35						

A rational approach to setting MRLs would be to interpolate the tolerance limits values for a withdrawal time between 3 and 7 days on the basis of a complete data set for all tissues. The official withdrawal times for the products registered in the four countries 1 were 10 (1 country) to 12 (3 countries) days. To base the MRLs on withdrawal times > 7 days is difficult because valid quantitative data for the marker residue in muscle and skin/fat are not available.

It seems possible to determine the ratio of marker to total residue concentrations by an alternative approach, namely by dividing the values of the two regression lines (the present marker residue study T5C619610 and the total residue study T5C749602 for all given time points for which they are valid. However, in this case the results of the total residue study have to be adjusted taking into account the 1.43 fold higher dose in the marker residue study. The following ratios – given in table 14 - are then obtained:

Table 14: Alternative to estimate the chicken marker to total residue concentrations.

day	Liver	Kidney	Muscle	Skin fat
	Ratio of the values of the depletion curves for marker and total residues			
3	0.67	0.62	0.91	0.53
7	0.53	0.22	1.25	0.45
10	0.45	0.10		
14	0.35			
17	0.30			

The results are in reasonable agreement with the results of study T5C749602 for liver if one takes into account all uncertainties. For the other tissues the values given in table 14 are possibly the more reliable estimates and can be used for the intake assessment with turkey tissues for which no total residue study is available. However, for EDI estimates with chicken tissues it seems to be most appropriate to directly use the total residue study after adjustment of the values as described above.

If only the EDI < ADI criterion is examined, then MRLs could be based on the tolerance limits observed on day 3 after treatment or later. Using the above mentioned adjustment factor of 1.43 the EDI values calculated in table 11 would change as given in table 15.

Table 15: Chicken EDI estimates adjusted to the dose range of the marker residue study.

day	Liver	Kidney	Muscle	Skin fat	All tissues	% of ADI
	EDI [µg/person/day]					
3	419	62	47	10	538	22.4
7	223	47	17	4	291	12.1
10	139	38	38	2	187	7.8

The ADI is numerically also the microbiological ADI for this substance. It is therefore desirable to ensure that occasional high intakes to be expected due to the high variability of the data also remain below the ADI with reasonable statistical certainty.

A computer modelling exercise was carried out in which on the basis of normally distributed random numbers and the kinetic parameters obtained from regression analysis of the logarithms of the residue concentrations 29220 “food packages” were generated. This number corresponds to 80 years of human life. From the results which are summarised in table 16 the recommended MRLs should not be based on three days withdrawal time because in this case approximately up to 2.5 % of calculated intakes would exceed the ADI. By using the data of day 7 this frequency could be reduced to < 0.3 %. Statistically based MRLs cannot be set for kidney, muscle and skin/fat for withdrawal periods beyond 7 days. Table 16 also shows that for this study the results for the median intake of the computer modelling and the calculated EDI are within 0.6 % identical.

Table 16: Comparison of the results of computer modelling of intakes and of the chicken EDI calculation.

Withdrawal time [days]		3	4	5	6	7	7	7	7	7	7	7	7	7	7
Upper class limit expressed as:						Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6	Trial 7	Trial 8	Trial 9	Trial 10
% ADI	µg/ day	**Cumulative frequency [%]**													
10	240	6.1	10.8	16.8	25.0	33.6	33.6	33.1	33.6	34.0	34.3	33.5	33.8	33.9	34.0
20	480	38.8	48.6	58.6	66.8	74.4	74.0	74.7	74.2	74.4	74.4	74.7	74.6	74.8	74.6
30	720	64.0	71.5	79.1	84.2	88.9	88.4	88.7	88.5	89.0	89.1	89.0	89.0	89.0	89.0
40	960	78.1	83.2	88.5	91.7	94.6	94.4	94.4	94.4	94.6	94.6	94.7	94.7	94.5	94.5
50	1200	85.7	89.8	93.1	95.2	97.1	97.0	97.1	97.1	97.1	97.2	97.2	97.2	97.1	97.0
60	1440	90.1	93.4	95.7	97.1	98.4	98.3	98.4	98.3	98.4	98.4	98.4	98.4	98.3	98.4
70	1680	93.6	95.7	97.3	98.3	991	99.0	99.1	99.1	99.1	99.1	99.1	99.1	99.0	99.1
80	1920	95.6	97.0	98.2	98.8	99.4	99.3	99.5	99.5	99.4	99.5	99.4	99.4	99.4	99.4
90	2160	96.8	97.9	98.8	99.2	99.6	99.6	99.6	99.6	99.6	99.6	99.7	99.6	99.6	99.6
95	2280	97.2	98.3	99.0	99.4	99.7	99.7	997	99.7	99.7	99.7	99.7	99.8	99.7	99.7
100	2400	97.6	98.5	99.1	99.5	99.8	99.7	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.7
200	4800	99.8	99.9	100.	100.	100.	100.	100.	100.	100.	100.	100.	100.	100.	100
300	7200	100.	100.	100	100	100	100	100	100.	100.	100.	100.	100	100.	100
Lowest intake [µg]:		71	71	73	58	45	39	42	47	47	44	38	49	44	40
Median intake [µg]:		571	492	417	357	312	311	310	309	308	308	311	310	308	308
Highest intake [µg]:		14192	7717	7508	7623	5290	5857	7272	6394	5021	9844	7147	5523	6293	4259
EDI	Liver	419.0				222.7									
	Kidney	62.4				47.2									
	Muscle	47.2				17.5									
	Skin/fat	10.5				4.2									
	Basket	539				292									

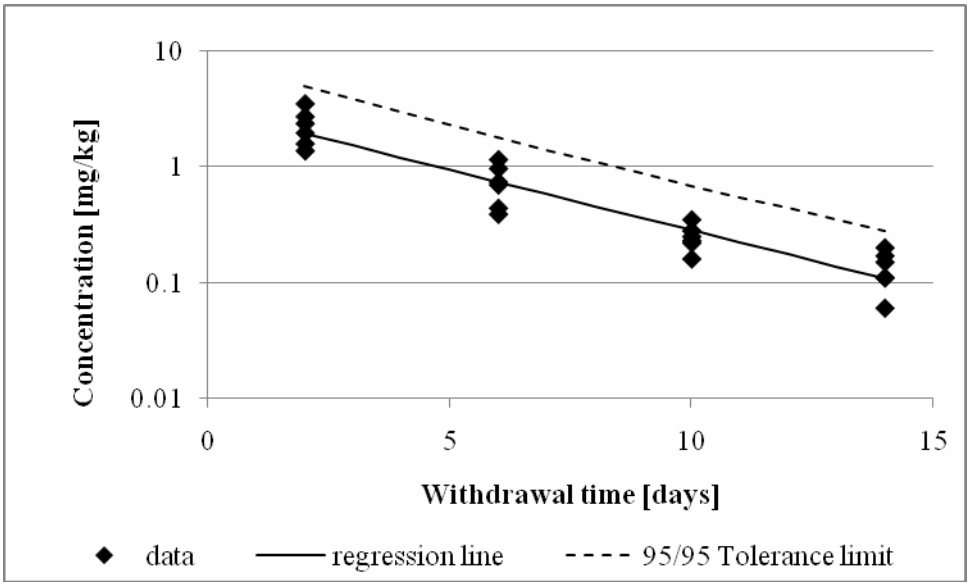
Turkey

In a GLP compliant study (TUR – 99 – 10, Warren, 2000) grower turkeys, 7-8 weeks of age were given continuous access *ad libitum* over 72 hours to medicated drinking water containing 75 mg/L of tilmicosin. Water consumption was given only on a pen basis. The average body weight of the animals on day 0 was 3.42 kg with a range from 2.58 to 4.56 kg. An average daily dose of 9.9 mg/kg bw was calculated (range from 9.6 to 10.5 mg/kg bw/day). According to the label, the Irish authority expects a dose range of 6 – 30 and the French authority a dose range of 10 – 27 mg/kg bw/day resulting from the recommended treatment. Thus the study ranges are at the lower end of the expected dose range.

Three male and three female birds were sacrificed 2, 6, 10, 14, and 18 days after cessation of treatment. Residue data were provided for liver, kidney, skin/fat and muscle. A validated HPLC

method was used for the determination of tilmicosin. In liver quantifiable concentrations of residues were observed from day 2 to 14. In kidney, samples of two female animals were below the limit of quantification. For statistical evaluations half the limit of quantification was used for these samples. The situation was similar for skin/fat. In muscle quantifiable results were only obtained in samples of days 2 and 6. Compared with the chicken marker residue study doses were less variable and also the variability of the residue data was much smaller. An example of the results of statistical treatment of the data is given in figure 13 below.

Figure 13: Statistical evaluation of residue data for liver of turkey.



When linear regression analysis was performed in a semi-logarithmic system (logarithms to the base 10), the following parameters (table 17) were obtained (where "a" is the log of the extrapolated concentration at zero withdrawal time, "b" is a measure of the depletion rate constant and $s_{y.x}$ is the residual variance. Analysis shows that in liver of turkey the initial concentrations were slightly higher compared to chicken liver. In muscle the two concentrations were similar and in fat and muscle concentrations were lower in turkey compared to chicken. However, the rate of depletion was higher in chicken with the exception of liver in which the depletion rate in turkey was higher. The residual variance in chicken was significantly higher, possibly due to the high variability in the doses found in chicken studies.

Table 17: Comparison of statistical parameters for chicken and turkey tissues.

Turkey

Parameter	Liver	Kidney	Skin/Fat	Muscle
a:	0.49379	0.25866	0.60122	0.75071
b:	-0.10411	0.10022	0.06573	0.08942
$s_{y.x}$:	0.16467	0.21830	0.17113	0.12490
n	24	24	24	12

Chicken

a:	0.54487	0.16678	0.77698	0.74695
b:	-0.09394	0.14458	0.07329	0.11919
$s_{y.x}$:	0.26726	0.29110	0.26670	0.31530
n	47	30	20	20

These results do not support the same MRLs in turkey and chicken tissues. The figures, 14a and 14b, support this observation by visualizing the regression lines obtained for the two species and the four

tissues. MRLs should be recommended on the basis of seven day withdrawal time. The practical withdrawal time to comply with these limits could be longer if the dose range observed in practice is in fact higher than the one used in the study TUR – 99 – 10. For this time point the following values for the median value and the tolerance limits have been obtained by statistical data analysis:

Figure 14: Comparison of a) regression lines and b) tolerance limits for chicken and turkey tissues.

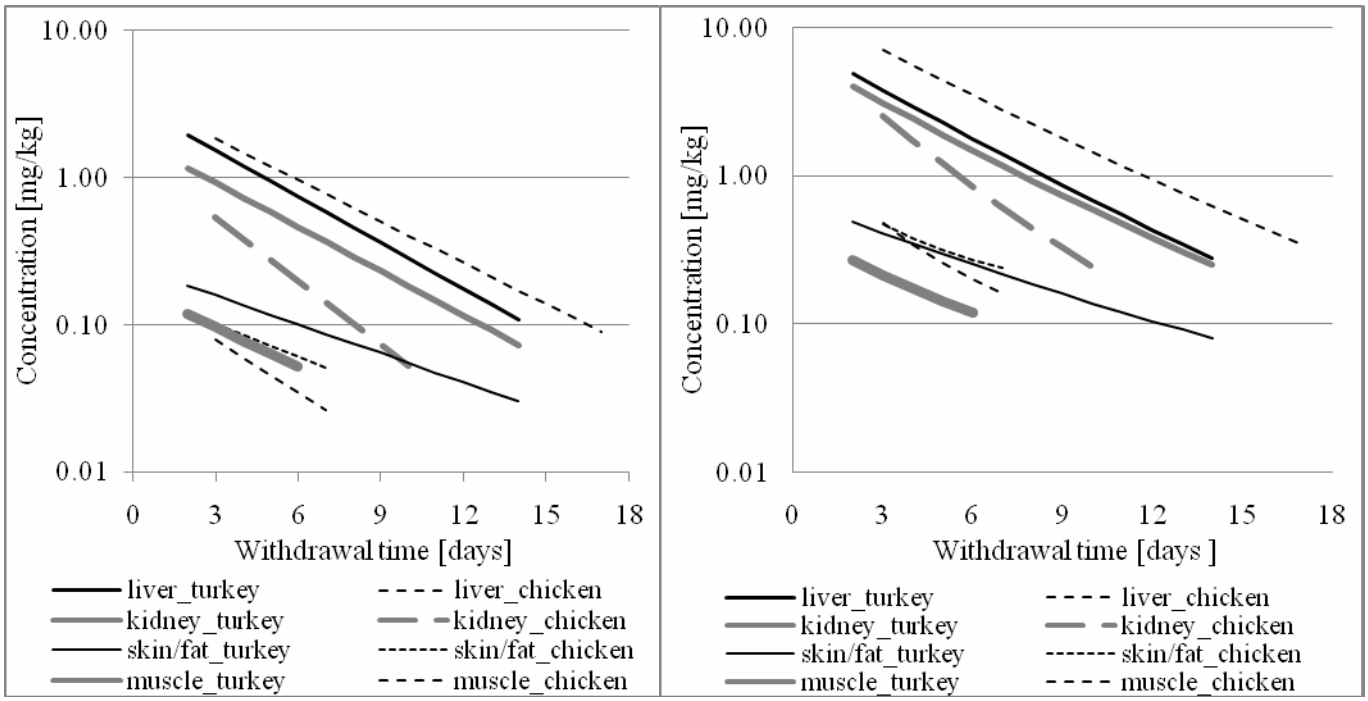


Table 18: Basis for recommending MRLs in turkeys.

day	Liver		Kidney		Skin/fat		Muscle	
	median	Tol.-limit	median	Tol.-limit	median	Tol.-limit	median	Tol.-limit
7	0.582	1.400	0.361	1.154	0.087	0.216	0.042	0.101

The following factors could be used for the conversion of marker to total residue concentrations: liver 0.5, kidney 0.25, skin fat 0.45, and muscle 1.0.

Chicken eggs

Study with ^{14}C-labelled tilmicosin

In the study 004-00780 mentioned previously the hens received daily for three consecutive days oral doses via gavage of 19.1 ± 0.1 mg/kg bw of ^{14}C-tilmicosin as two divided doses in the morning and in the evening. The initial body weights of the hens (day -1) ranged from 1.166 to 1.463 kg. The total number of eggs produced per animal and within the study days 0-23 ranged from 20-24. Table 19 summarizes the animal data. Animal V19 produced the lowest number of eggs including one soft-shelled egg, and the lowest amount of egg material during the 24 days observation period. Concentrations of residues were highest in egg white and egg yolk of this animal on every day.

Table 19: Egg and animal data.

Animal ID	bw [kg]	Daily Dose [mg/kg bw]		Weight [g]			Amount of residues [µg]		
			Number of eggs	Egg white	Egg yolk	Total egg content	In egg white	In egg yolk	In total egg content
V10	1.25	20.0	23	768	322	1089	735	249	984
V12	1.37	20.0	24	714	339	1053	580	228	808
V14	1.39	20.0	23	759	320	1078	712	278	990
V17	1.39	20.0	24	853	354	1207	501	219	720
V19	1.26	19.9	20	631	285	916	2445	999	3444
V22	1.17	19.8	22	605	284	890	505	305	811
V24	1.37	19.8	21	825	386	1211	539	218	758
V25	1.46	19.8	24	936	382	1317	832	233	1065

Figure 15 shows the kinetics of depletion of total radioactive residues in total egg content. The concentrations in egg white and in egg yolk were in the same order of magnitude. The ratio of the concentrations in egg white and in egg yolk was 1.24 ± 0.41. The median concentration in total egg content reached a peak of 4.2 mg/kg on day 3. The maximum of 13.7 mg/kg was observed in an egg of animal V19 on day 4. The concentrations of residues are not normally distributed. If one assumes a log-normal distribution, the values obtained with animal V19 fall within 3 standard deviations of the geometric mean and should not be excluded from calculations.

Marker residue and ratio marker to total residue

Pools of all egg whites and egg yolks (except from animal V19) from days 3, 7, 11, and 18 were formed. The report states that equivalent masses were taken from each egg. The corresponding samples from animal V19 were analysed separately. Samples were twice extracted with acetonitrile. The remaining pellet is called “nonextracted” in table 20 below and expressed in percent. The extract was further cleaned and analysed by HPLC-MS/MS. The authors provide the concentrations of tilmicosin and T-12 on the basis of the initial sample mass. However, the percent of total radioactivity is calculated on the basis of the radioactivity in a given peak and total radioactivity injected onto the column. This approach overestimates the ratio. It is better to base the ratio of marker to total residue concentrations on the basis of the mass of the samples in order to take account of the residues remaining in the pellet. This approach slightly underestimates the ratio since the unknown recoveries cannot be taken into account; however, it seems appropriate to follow the more conservative approach. The results of the calculations are given in table 20. The values obtained for the pools established from eggs collected on day 18 are outside the range of all other values. It is proposed not to use these results, in particular since intake estimates for such late time points of the depletion kinetics will not be made. For the early time point a value of 0.7 for the ratio of marker to total residue concentrations is sufficiently conservative.

Figure 15: Depletion of residues of ^{14}C-tilmicosin in eggs.

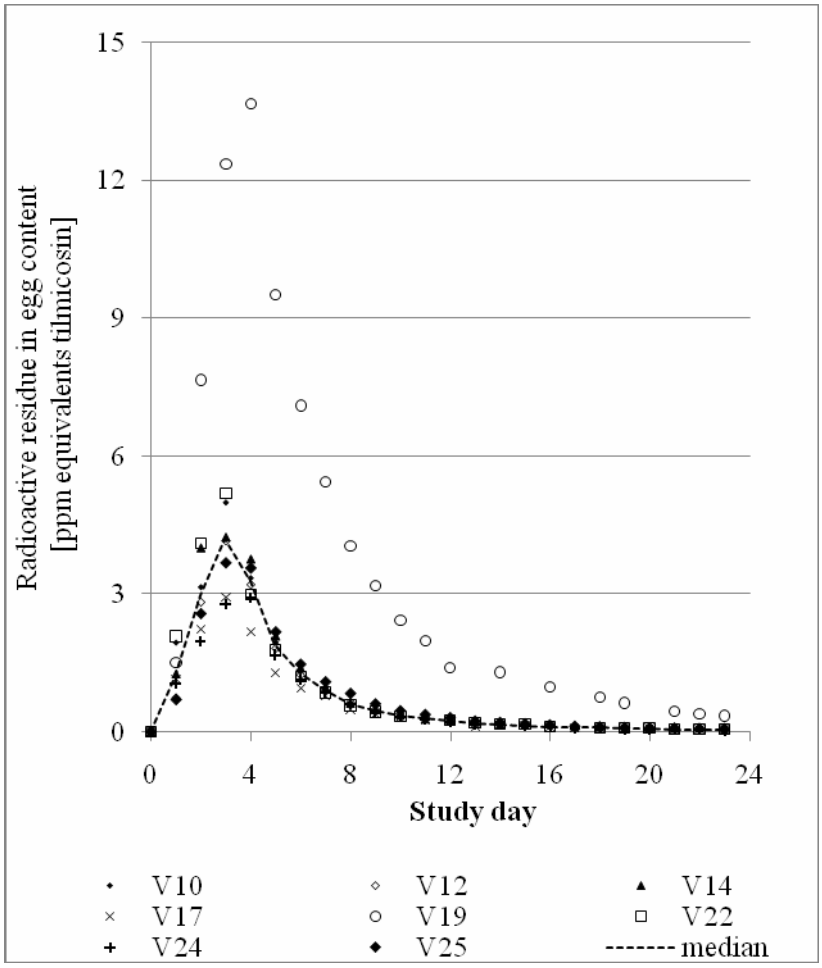


Table 20: Ratio of marker to total residue concentrations.

day	Calculated total residue [mg/kg]	% Not extracted	Tilmicosin [mg/kg]	T-12 [mg/kg]	Ratio	Measured total residue[mg/kg]	% not extracted	Tilmicosin[mg/kg]	T-12[mg/kg]	Ratio
3	4.10	4.9	3.04	0.10	0.74	12.92	5.2	8.34	1.12	0.65
7	0.90	4.0	0.58	0.04	0.65	5.51	4.4	3.46	0.52	0.63
11	0.32	4.1	0.21	0.03	0.66	2.03	4.8	1.26	0.23	0.62
18	0.10	5.6	0.15	0.02	1.42	0.85	5.4	0.51	0.12	0.60
3	3.76	6.5	2.84	0.03	0.76	11.09	6.9	7.74	0.23	0.70
7	0.97	7.4	0.64	0.01	0.65	5.29	6.7	3.48	0.15	0.66
11	0.26	9.2	0.18	0.01	0.68	1.89	8.3	1.34	0.07	0.71
18	0.09	9.3	0.10	0.00	1.16	0.59	12.5	0.36	0.02	0.62

Study with unlabelled tilmicosin

In a GLP compliant study (004-00781, Beauchemin, et al. 2007b), fifteen hens of an approximate age of 41 weeks and a body weights of 1.59 to 2.15 kg were dosed for three days via drinking water. Dose

amounts were calculated based on study day (-1) body weights. The targeted dose was 15 to 20 mg/kg bw/day. The average calculated dose was 17 mg/kg bw/day. The individual doses per animal and day are not given. Information on registered doses is not available since all label copies provided by the sponsor warn that tilmicosin should not be used in birds producing eggs for human consumption. The light/dark cycle was set to 17 hours of light and 7 hours of dark. Two animals did not drink much of the treated water and had decreased egg production. One of these animals was treated as an outlier and excluded from data analysis. The data were used from the other animal (ID 270).

Eggs were collected from day (-1) to day 23. Some animals produced two eggs on a day (animal 293/day 0; animal 265/day 3; animal 270/day 11). In these cases the two eggs were combined into one sample. Weights of the egg contents were not given. Egg contents were analysed only for the odd days of the study. Therefore, for some animals the highest observed concentration may not represent the peak concentration. HPLC-MS/MS was used for analysis. Table 21_summarises animal-related data. Figure 16 shows the quantified results above the LOQ on a double linear scale.

The residue concentrations found are not normally distributed. Several alternative quantitative evaluations of the data are discussed. In the first two alternatives, the logarithms of the concentrations are used. A mean, a standard deviation, and an upper 95% confidence limit of the 95th percentile is calculated for each time point on the basis of the logarithms. The calculation was performed once including the data of animal 270, and once excluding the data. Since the sample size is very small and the variability of the results is extreme, the tolerance limits are very high. The results are given in table 22. The last column shows the results obtained if the data of animal 270 were not used.

Table 21: Animal body weights and egg production.

Animal ID	Body weight on day [-1) [kg]	Number of eggs produced from day 0-23
252	1.80	21
253	1.70	20
254	2.15	22
255	2.05	21
257	2.11	22
264	1.75	24
265	1.87	24
267	1.73	21
268	1.86	24
270	1.72	21
272	1.89	23
277	1.68	19
289	1.87	22
293	1.63	23

In figure 16, the data of the hens producing eggs with the lowest (270) and the highest (267) concentrations of residues are connected by a dotted line.

Figure 16: Depletion curves of marker residue in total egg content.

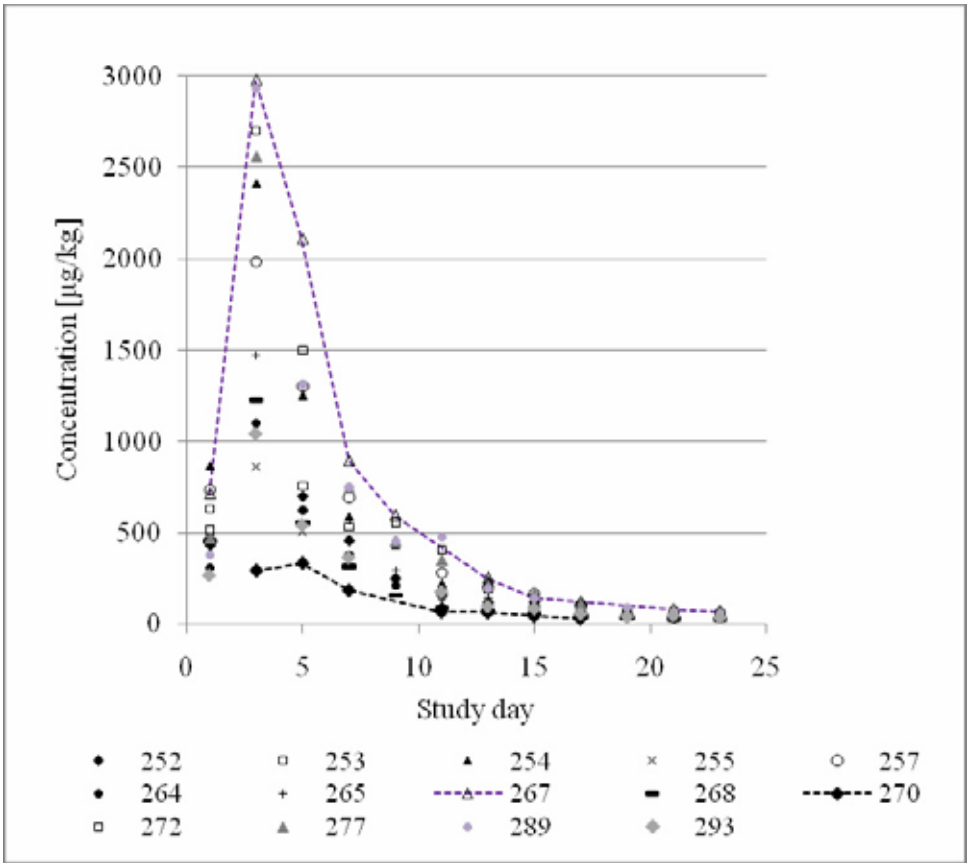


Table 22: Statistical evaluation of the laying hen eggs data.

Study day	n	mean (log scale)	s.d. (log scale)	k	Tolerance limit (Mean + k x s.d.)	Antilog mean	Antilog Tolerance limit	Antilog Tolerance limit excluding animal 270
		[dimension-less]				[µg/kg]		
1	13	2.56999	0.46595	3.081	4.00559	372	10130	10130
3	12	3.18108	0.29633	3.162	4.11807	1517	13124	7980
5	12	2.92075	0.23969	3.162	3.67864	833	4771	4504
7	12	2.69558	0.19536	3.162	3.31330	496	2057	1633
9	10	2.52326	0.19621	3.379	3.18626	334	1536	1536
11	13	2.25325	0.26589	3.081	3.07244	179	1182	1138
13	13	2.14317	0.20244	3.081	2.76688	139	585	558
15	13	2.02723	0.18442	3.081	2.59543	106	394	338
17	13	1.84576	0.18193	3.081	2.40628	70	255	221
19	10	1.66248	0.23934	3.379	2.47120	46	296	168
21	13	1.67755	0.22138	3.081	2.35963	48	229	150
23	13	1.62244	0.19941	3.081	2.23681	42	173	173

A plot of the same data on a semi-logarithmic scale system would show that the results obtained within days 3 and 15 follow roughly a linear pattern. The sponsor proposes to carry out a statistical analysis on this basis using linear regression. This approach is not appropriate since the eggs are obtained every day from the same hens. If the product would be registered for use in laying hens, the tolerance limits calculated in a table of the type of table 22 would form the basis for the calculation of MRLs. However, in the present case an MRL cannot be proposed because the number of animals used in the study is too small to adequately assess the great variability of the residue concentrations. The amount of data was further reduced because the only eggs of every second day were analysed and – on the present limited data base - one cannot exclude that the egg discard times required to ensure an acceptable distribution of daily intakes are not practicable. Furthermore it cannot be judged whether the dose regimen was adequate because the product is not registered for use in laying hens.

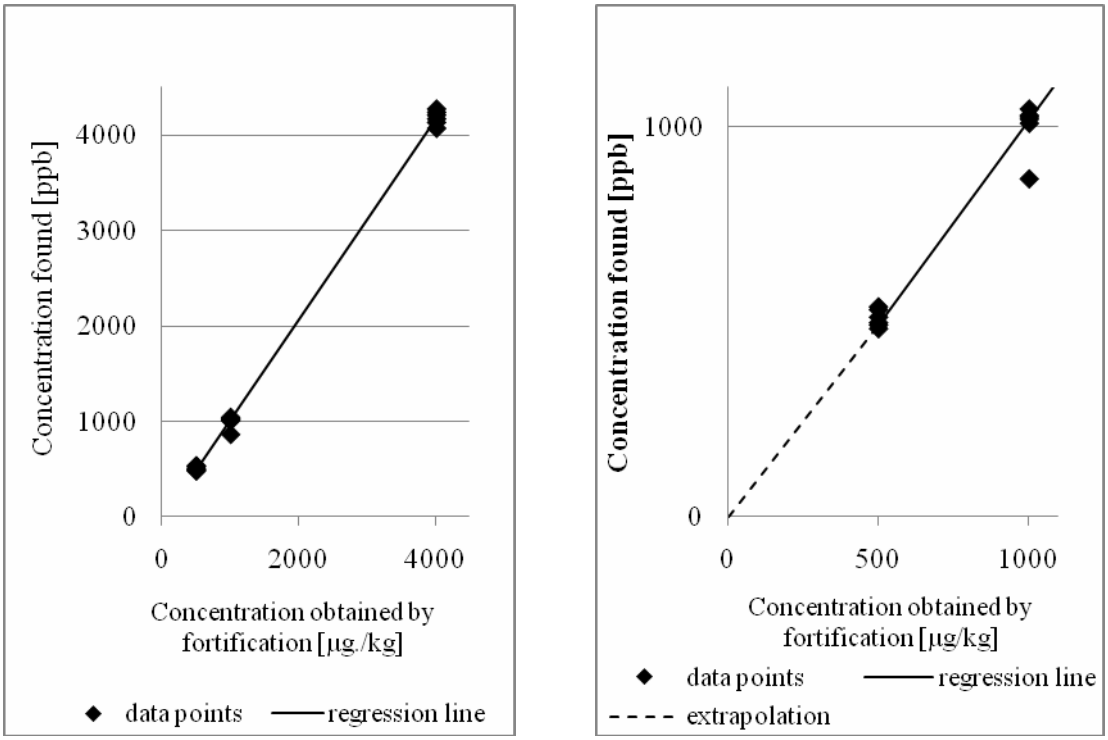
Rabbits

A tilmicosin tissue residue study (RTC study 6483, Luperi and Brightwell, 1999a) was conducted in the rabbit. Test animals were New Zealand White Rabbits of a body weight range of 1890 – 2150g for the males and 1924 – 2200g for the females. Animals received a single subcutaneous injection of tilmicosin calculated to result in a 10 mg/kg bw dose. The only example of a registered use of tilmicosin in rabbits recommends oral administration in the feed on the basis of a granulate and the doses vary depending on the indication between 5 – 6 and 10 – 12 mg/kg bw.

In the present study five animals (at least two of each sex) were sacrificed after various withdrawal times (7, 14, 21, 28 and 35 days) and the contents of parent drug tilmicosin were determined in liver, kidney, abdominal fat and muscle (tissue from the semimembranosus and semitendinosus muscle). Injection sites were excised in a portion of tissue of the trapezius and longissimus thoraci muscle of approximately 36 – 54g and were analysed for tilmicosin. The method used involved HPLC separation and UV detection at 280 nm. The method was only partially validated (Luperi and Brightwell, 1999b) using the following concentrations of tilmicosin obtained by fortifying blank tissues: muscle, 125.5, 251 and 1004; liver and kidney, 502, 1004 and 4016; fat, 25.1, 50.2 and 200.8 µg/kg, respectively.

The concentrations of all incurred tissues except 4 kidney samples and two fat samples were outside the range of concentrations for which the method was validated. At all the above given concentrations the method did fulfil the required accuracy and precision criteria. The authors declared the lowest concentration used in the validation study the limit of quantification though this is not often the case. When it happened that the incurred concentrations were lower, the analytical curve was extrapolated down to the origin of the coordinate system though this is not a good practice. The concentrations determined by this way were reported quantitatively – if they were above the limit of detection, but were labelled with an asterisk if they were below. The following figure 17 describes the approach on the example of liver. The left part shows the analytical curve obtained in the validation study. The right part explains at higher magnification the extrapolation procedure.

Figure 17: Use of analytical curves in the residue study in rabbits.



Normally, this approach would not be acceptable. However, it could not be excluded that the reported residue concentrations represented valid data and only the LOQ had been inadequately estimated. The problem was discussed with the sponsor in order to explore the possibility of a solution, but the sponsor confirmed that they only supported the use of the data above the limit of quantification.

The limit of detection was determined from the average plus three standard deviations obtained from the analyses of 21 blank tissues. It is not reported whether these were 21 independent tissues or 21 replicate determinations of one tissue. The sponsor could not answer this question, but was assuming that the 21 samples were replicate of the same composite sample composited from different animals.

Residues above the limit of detection of 3.5 µg/kg were not found in any sample of muscle. Residues at injection sites were below the limit of detection of 3.5 µg/kg in all samples collected at and after 14 days. Residues in fat were above the limit of detection of 3.2 µg/kg in all samples collected on day 7 and in about 50% of the samples obtained on days 14 and 21. In liver, residues above the limit of detection were found in all samples until 14 days after treatment and in three of five samples collected on day 21. Kidney was the organ with the highest concentrations found. All samples obtained until day 28 and one sample of an animal sacrificed on day 35 contained tilmicosin in concentrations above the limit of detection of 0.78 µg/kg. Figures 18 and 19 demonstrate the problems of the data base in view of the method validation data of the study.

Figure 18: Relationship of the measurements in liver of rabbits to the LOQ.

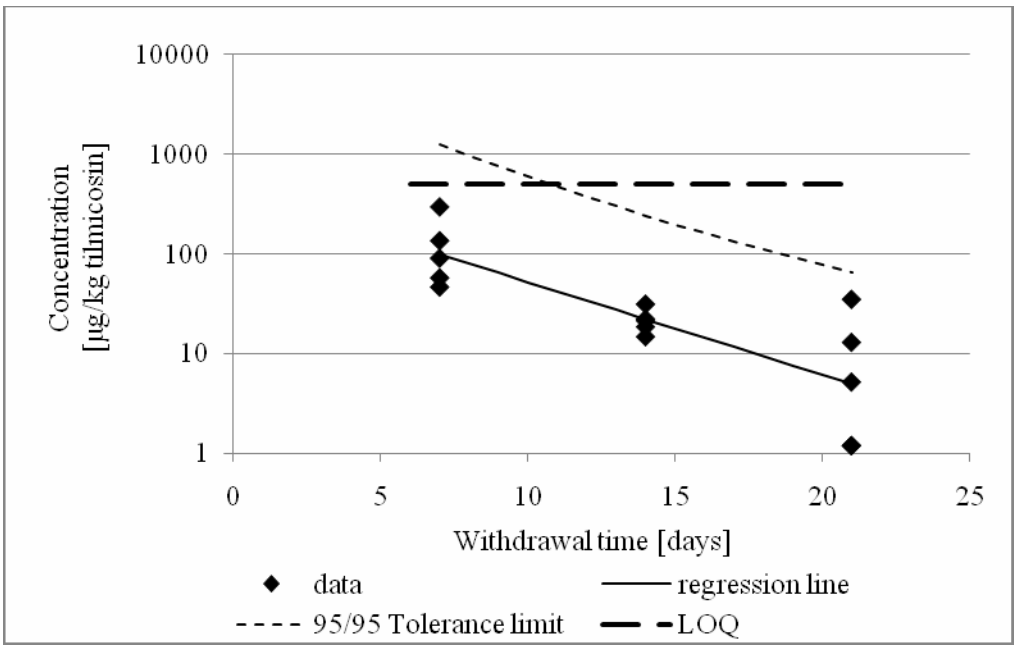
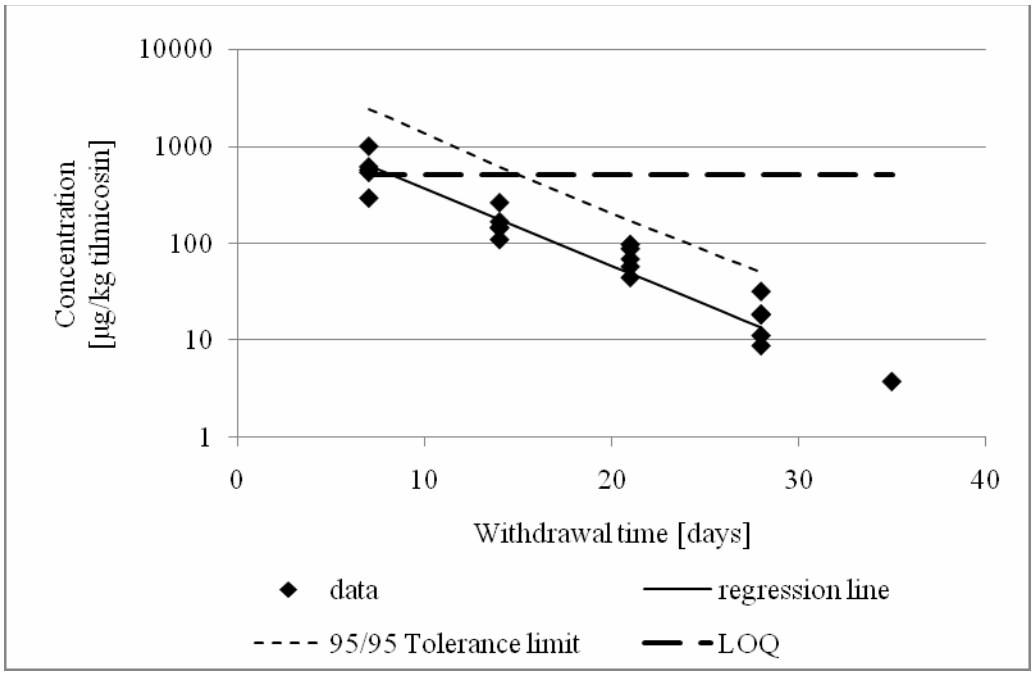


Figure 19: Relationship of the measurements in kidney of rabbits to the LOQ.



The ratio of marker to total residue is not known for rabbit tissues. The basic pattern of metabolites found in other species has been qualitatively confirmed by Montesissa, et al. (2004) using primary hepatocyte cultures and liver microsomes from rabbits and LC-MS methods for the identification of the metabolites. The data base provided by the sponsor is not suitable for recommending MRLs.

ESTIMATION OF DAILY INTAKE

All intake estimates were based on the information obtained from kinetic residue depletion studies. Three approaches were followed.

- For residues of tilmicosin in chicken the EDI was calculated directly from the total residue study at the same time point (7 days) on which the estimation of MRLs was based. The results are summarised in table 23.
- In a second approach, a computer modelling exercise was carried out in which on the basis of normally distributed random numbers and the kinetic parameters obtained from regression analysis of the logarithms of the residue concentrations 29220 "food packages" were generated. This number corresponds to 80 years of human life. The results showed that at 7 days withdrawal time the frequency of occurrence of above ADI "food packages" was below 0.3%. The modelling also showed that for this study the results for the median intake of the computer modelling and the conventionally calculated EDI were within 0.6 % identical. The results are presented in table 16.
- The third approach was applied to estimate intakes resulting from the consumption of turkey tissues. It was the conventional approach involving median marker residue concentrations and factors to adjust for the ratio of marker to total residue concentrations. The factors obtained for chicken were used for turkey tissues. The results are summarised in table 24.

Table 23: Estimate of chronic intake derived from total residue study in chickens on day 7.

	Liver	Kidney	Muscle	Fat/skin	All tissues
Predicted median concentration of total residue equivalents [µg/kg] on day 7 after treatment	2227	943.8	58.3	83.1	
Daily amount consumed [kg]	0.1	0.05	0.3	0.05	0.5
Daily intake of total residue equivalents	223	47	18	4	292
% of upper limit of ADI	9.3	2.0	0.7	0.2	**12**

Table 24: Estimate of chronic intake derived from marker residue study in turkeys on day 7.

	Liver	Kidney	Muscle	Fat/skin	All tissues
Predicted median concentration of marker residue concentration [µg/kg] on day 7 after treatment	582	361	42	87	
Daily amount consumed [kg]	0.1	0.05	0.3	0.05	0.5
Daily intake of marker residue [µg/kg]	58	18.0	13	4	
Conversion factor marker to total	1/0.5	1/0.25	1	1/0.45	
Daily intake of total residue equivalents [µg/kg]	116	72	13	10	211
% of upper limit of ADI	4.9	3.0	0.5	0.4	**8.8**

METHODS OF ANALYSIS

A validated HPLC method was provided to analyse tilmicosin in edible tissues of several species including chicken and turkey tissue (Lilly Method B04228 rev 7). It is based on a solid-phase extraction, gradient elution and UV detection. It was validated for chicken tissues as to linearity, precision, accuracy, specificity, ruggedness, and stability of tilmicosin. The modification for turkey tissues was validated for the same criteria in an additional study (Hawthorne, 1999). The LOQ is 60µg/kg for liver and kidney and 25µg/kg for muscle and fat.

An LC/MS-MS method was provided to analyse tilmicosin in whole egg with a LOQ of 25 µg/kg (MPI Method V0003516). It was validated according to U.S. FDA guidelines (McCracken, 2007).

A validated HPLC method, based on a solid-phase extraction, gradient elution and UV detection is available to analyse tilmicosin in cow and sheep milk with a LOQ of 10µg/kg (Method B05704, Revision 3). A validation document for this method was also provided. Tilmicosin residues can be detected in milk using commercial bacterial growth inhibition test.

APPRAISAL

The forty-seventh meeting of the Committee established an ADI of 0-40 µg/kg body weight (0-2400µg per day for a 60 kg person) and MRLs (µg/kg) for cattle, sheep and pigs were recommended in muscle, liver, kidney and fat tissues. A temporary MRL was recommended for sheep milk. The temporary MRL of 50 µg/kg for milk of sheep was not extended by the Committee at the fifty-fourth meeting because results of a study with radioactively labeled drug in lactating sheep to determine the relationship between total residues and parent drug in milk was not available. The present Committee addressed both new and relevant previously submitted data.

The sponsor requested the Committee to recommend MRLs for tilmicosin in chicken, turkey and rabbit tissues, chicken eggs and an MRL for milk of sheep. In this submission the sponsor explained the reasons for not having provided a total residue study in sheep milk using ^{14}C-tilmicosin as requested by the 47th JECFA. The sponsor proposed MRLs and provided deliberations about dietary intakes resulting from all uses of the products under conditions of compliance with the proposed MRLs.

In chickens, using radiolabel studies, the structure of metabolites was determined using ESP-MS. In total, a number of metabolites and parent tilmicosin were found in the extracts. The structures are briefly described in table 5. Studies suggest that in liver approximately 55% of the total radioactive residue represents parent tilmicosin. The corresponding values for kidney and muscle are approximately 40%. The highest residue concentrations were observed in liver followed by kidney. Residue concentrations in skin fat, abdominal fat and muscle were very low. No similar study was provided for turkeys.

Although tilmicosin is not recommended for production of eggs for human consumption, the sponsor provided data on residues in eggs using radiolabel studies. The ratio of tilmicosin to total residue was calculated and a value of 0.7 was estimated from the data base provided

Studies were also provided on milk from lactating dairy cows. Residues may persist for more than 50 days and tilmicosin represented up to 89 percent of the total radioactive residue in one study. The labels of registered products provided by the sponsors warn that tilmicosin should not be used in cows producing milk for human consumption.

The sponsor had been requested to provide a radiolabel study for consideration of an MRL in sheep milk but none was provided. Only limited residue studies were provided. Milk was analysed for parent tilmicosin using an HPLC method with a limit of quantification of 50 µg/l. The milk was also subjected to a Delvotest and full inhibition was found for the first 6 to 7 days. No inhibition in any sample was found after day 12. The data base of this study was very limited. The weaknesses of the study cannot be compensated by recommending high MRLs. Consumption of milk obtained within the first 144 hours after treatment likely leads to intakes exceeding the ADI.

A rational approach to recommending MRLs in chickens would be to interpolate the tolerance limits values for a withdrawal time between 3 and 7 days on the basis of a complete data set for all tissues. The registered withdrawal times based on provided labels for the products registered in the four countries were 10 (1 country) to 12 (3 countries) days. To base the MRLs on withdrawal times > 7 days is difficult because valid quantitative data for the marker residue in muscle and skin/fat are not available.

The sponsor proposed to carry out a statistical analysis on the egg studies using linear regression to recommend an MRL. This approach is not appropriate since the eggs are obtained every day from the same hens. However, in the present case an MRL cannot be proposed because the number of animals used is too small to adequately assess the great variability of the residue concentrations. Furthermore it cannot be judged whether the dose regimen was adequate because the product is not registered for use in laying hens.

In the rabbit studies, the concentrations of all incurred tissues except four kidney samples and two fat samples were outside the range of concentrations for which the analytical method was validated. The authors declared the lowest concentration used in the validation study as the limit of quantification though this is not often the case. When it happened that the incurred concentrations were lower, the analytical curve was extrapolated down to the origin of the coordinate system, though this is generally not a good practice.

MAXIMUM RESIDUE LIMITS

The Committee considered data for recommending MRLs in chicken, turkeys, eggs, rabbit and sheep milk. The sponsor provided information on registered uses, which showed that there is at present no registered use for laying birds. The residue concentrations in eggs were very high and could result in long withdrawal times.

In the rabbit, the residue depletion study was performed using subcutaneous administration. However, the registered oral use administration route was not covered by an adequate residue depletion study.

The argument of the sponsor that a radiolabelled residue in sheep milk was not necessary, as new data were provided to bridge between cattle and sheep, was accepted in principle. The only residue study in lactating ewes contained an insufficient number of animals to allow MRLs to be recommended and showed that long milk withdrawal times of approximately 15 days may be required.

For chickens, a satisfactory data set was available to derive MRLs. For turkeys, the available residue did not include a total residue study, but the data could be bridged by using ratios of marker to total residue concentrations derived from the study in chickens.

When recommending MRLs the Committee considered the following points:

- The ADI for tilmicosin was 0-40 µg/kg bw/day corresponding to an upper bound of acceptable intakes of 2400 µg per day for a person with a body weight of 60 kg.
- The time point on which the MRLs were set was based on an EDI < ADI approach *and* on modelling of possible intakes resulting from the consumption of the four standard edible tissues showing that > 99.7 % of all intakes in 80 years life time would be below the ADI.
- The residue depletion kinetics in turkeys were different from those found in chickens.
- The most suitable time point for the calculation of MRLs was 7 days after the end of treatment in chickens and turkeys.
- The studies provided clear evidence of dose-linearity of the residues in tissues of chicken.
- The range of therapeutic doses was covered by the studies performed with chickens. The dose used in the depletion study with turkeys was at the lower end of the registered dose regimes; however, the residue data from turkeys showed less than did the data from chickens.
- A total residue study in chicken could be directly used for the intake estimates following adjustment to account for the slightly higher range of therapeutic doses.
- The data from the marker residue study enabled statistical MRL calculations for chickens and for turkeys. MRLs were calculated on the basis of upper one-sided 95% confidence limits over the 95th percentile of residue concentrations.
- The ratio of marker to total residue concentrations was determined for chicken tissues and was applied for the estimated intakes of residues from turkey tissues.

- Data submitted to support MRLs for rabbit tissues, chicken eggs and sheep milk were not suitable to derive MRLs compatible with the registered conditions of use for tilmicosin.
- A validated method of analysis was available for chicken and turkey tissues.

The Committee recommended MRLs, determined as tilmicosin, as follows:

	MRLs [µg/kg]			
	Liver	Kidney	Muscle	Skin/Fat
Chicken	2400	600	150	250
Turkey	1400	1200	100	250

The Committee was not able to recommend an MRL for sheep milk.

Before a re-evaluation of tilmicosin with the aim to recommend MRLs in tissues of rabbits, the Committte would require adequately designed residue studies with doses and routes of administration under authorized conditions of use and using a validated method suitable for the purpose.

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Peters, A.R., Mead, G.G., and Wathes, D.C. (1997). Tilmicosin pharmacokinetic study in chickens following oral administration via the drinking water. Unpublished study from The Royal Veterinary College, University of London, Department of Farm Animal and Equine Medicine and Surgery, Potters Bar, Herts, England. Sponsor submitted.

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Readnour, R.S., Fossler, S.C., Stobba-Wiley, C.M., and Abrams, S.A. (1997). Tilmicosin drinking water medication residue decline study in shickens. Study T5C619610. Unpublished study from Animal Science Product Development, Elanco Animal Health, a division of Eli Lilly and Company, Greenfield, IN, USA. Sponsor submitted.

Stoev, G., and Nazarov, V. (2008). Identification of the related substances of tilmicosin by liquid chromatography/ion trap mass spectrometry. Rapid Communications in Mass Spectrometry, 22, 1993 – 1998.

Xu, Z., Wang, J., Shen, W., and Cen, P. (2006). Study on the extraction equilibrium of tilmicosin between the aqueous and butyl acetate phases. Chemical Engineering Communications, 193, 427-437.

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TRICLABENDAZOLE

First draft prepared by
Philip T. Reeves, Canberra, Australia
and
Gerald E. Swan, Pretoria, South Africa

Addendum to the monographs prepared by the 40th and 66th meetings of the Committee and published in FAO Food & Nutrition Paper 41/5 and FAO JECFA Monographs 2, respectively.

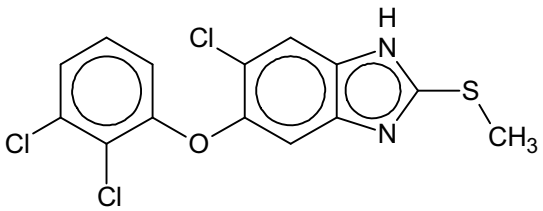
IDENTITY

Chemical name: 5-Chloro-6-(2,3-dichlorophenoxy)-2-methylthio-1H-benzimidazole {International Union of Pure and Applied Chemistry name}

Chemical Abstracts Service (CAS) number: 68786-66-3

Synonyms: Triclabendazole (common name); CGA 89317, CGP 23030; proprietary names Fasinex®, Soforen®, Endex®, Combinex®, Parsifal®, Fasimec®, Genesis®, Genesis™ Ultra.

Structural formula:



Molecular formula: $C_{14}H_9Cl_3N_2OS$

Molecular weight: 359.66

OTHER INFORMATION ON IDENTITY AND PROPERTIES

Pure active ingredients: Triclabendazole

Appearance: White crystalline solid

Melting point: 175-176°C (Merck), α-modification; 162°C, β-modification

Solubility: Soluble in tetrahydrofuran, cyclohexanone, acetone, iso-propanol, n-octanol, methanol; slightly soluble in dichloromethane, chloroform, toluene, xylene, ethyl acetate; insoluble in water, hexane.

RESIDUES IN FOOD AND THEIR EVALUATION

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) reviewed triclabendazole at its 40th and 66th meetings (FAO/WHO, 1993, 2006). At the 40th meeting the Committee established an ADI of 0-3 µg/kg of bodyweight (0-180 µg per day for a person of 60 kg bodyweight) and recommended the following Maximum Residue Limits (µg/kg):

Species	MRLs recommended by the 40th JECFA (µg/kg)			
	Muscle	Liver	Kidney	Fat
Sheep	100	100	100	100
Cattle	200	300	300	200

The FAO Food Nutrition Paper residue monograph prepared at the fortieth meeting (FAO, 1993) states: "The marker residue for triclabendazole is 5-chloro-6-(2', 3'-dichlorophenoxy)-benzimidazole-2-one and is produced when common fragments of triclabendazole-related residues are hydrolysed under alkaline conditions at 90-100°C… …Marker residue levels can be converted into triclabendazole equivalents by multiplying by a conversion factor of 1.09." In the report from the fortieth meeting of the Committee (FAO/WHO, 1993), it is noted in Annex 2 that the MRLs are expressed as 5-chloro-6-(2', 3'-dichlorophenoxy)-benzimidazole-2-one.

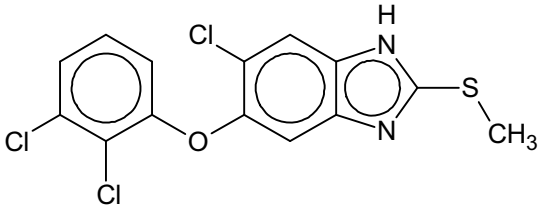
The 66th meeting defined the marker residue as "keto-triclabendazole" and recommended the following Maximum Residue Limits (µg/kg):

Species	MRLs in Tissues (µg/kg)			
	Muscle	Liver	Kidney	Fat
Cattle	150	200	100	100
Sheep	150	200	100	100
Goat	150	200	100	100

The sponsor (correctly) defined the marker residue as "sum of the extractable residues that may be oxidised to keto-triclabendazole" and proposed MRLs below as consistent with withdrawal periods of 35 days after oral administration to cattle and 27 days after oral administration to sheep and goats.:

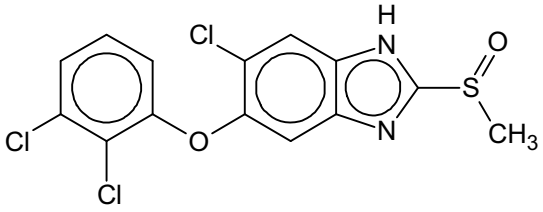
Species	MRLs in Tissues (µg/kg)			
	Muscle	Liver	Kidney	Fat
Cattle	275	600	375	200
Sheep	275	600	375	200
Goat	275	600	375	200

Triclabendazole is 6-chloro-5-(2', 3'-dichlorophenoxy)-2-methylthio-1-*H*-benzoimidazole (CAS number 68786-66-3). Its structure and the structure of some compounds related to it (e.g., metabolites and conversion products) are given in the scheme below:



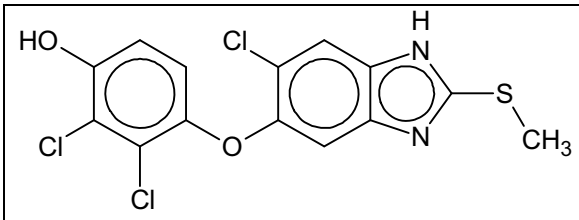
6-chloro-5-(2,3-dichlorophenoxy)-2-(methylthio)-1*H*-benzimidazole
Molecular Formula = $C_{14}H_9Cl_3N_2OS$
Formula Weight = 359.65
Synonyms and abbreviations are:
CGA 89 317 and CGP 23 030.

Triclabendazole



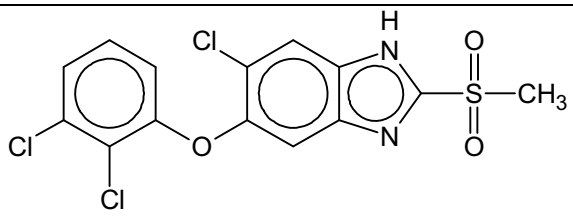
6-chloro-5-(2,3-dichlorophenoxy)-2-(methylsulfinyl)-1*H*-benzimidazole
Molecular Formula = $C_{14}H_9Cl_3N_2O_2S$
Formula Weight = 375.65
Synonyms and abbreviations are:
CGA 110 752

Triclabendazole sulphoxide



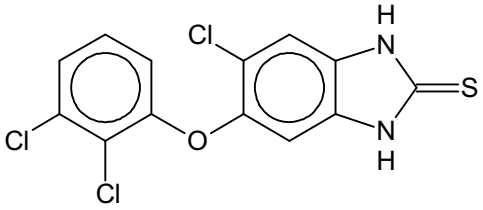
2,3-dichloro-4-{[6-chloro-2-(methylthio)-1*H*-benzimidazol-5-yl]oxy}phenol
Molecular Formula = $C_{14}H_9Cl_3N_2O_2S$
Formula Weight = 375.65
Synonyms and abbreviations are:
CGA 161 944

4-Hydroxytriclabendazole

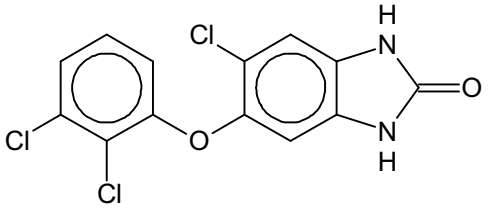


6-chloro-5-(2,3-dichlorophenoxy)-2-(methylsulfonyl)-1*H*-benzimidazole
Molecular Formula = $C_{14}H_9Cl_3N_2O_3S$
Formula Weight = 391.65
Synonyms and abbreviations are:
CGA 110 753

Triclabendazole sulphone



5-chloro-6-(2,3-dichlorophenoxy)-1,3-dihydro-2*H*-benzimidazole-2-thione
Molecular Formula = $C_{13}H_7Cl_3N_2OS$
Formula Weight = 345.63
Synomyms and abbreviations are:
CGA 77 336



5-chloro-6-(2,3-dichlorophenoxy)-1,3-dihydro-2*H*-benzimidazol-2-one
Molecular Formula = $C_{13}H_7Cl_3N_2O_2$
Formula Weight = 329.56
Synonyms and abbreviations are:
CGA 110 754

Keto-triclabendazole

Conditions of use

Triclabendazole is an anthelmintic used for the control of liver fluke, *Fasciola hepatica* and *F. gigantica*, in cattle, sheep and goats. Triclabendazole is contained in oral suspensions for cattle, sheep and, in some countries, goats as well as in pour-on formulations for cattle. Triclabendazole is also used for the treatment of fascioliasis in humans.

Dosage

Triclabendazole is administered to cattle as a drench at a nominal dose rate of 12 mg/kg of bw and as a pour-on application at a nominal dose rate of 30 mg/kg of bw. It is administered orally to sheep and goats at a nominal dose rate of 10 mg/kg of bw. Veterinary advice regarding the interval for repeat treatments differs from country to country; however, the recommended interval for routine treatment during the Fasciola season is reported to be 10 weeks.

PHARMACOKINETICS AND METABOLISM

Laboratory Animals

Rats

In a study conducted by Muecke (1981), two female and two male rats were each given a single oral dose of either 0.5 or 25 mg [^{14}C]-triclabendazole/kg of bw. The radioactive label was at the carbon atom in position 2 of the benzimidazole ring system. Radioactivity was determined by liquid scintillation counting. Urine was directly added to scintillation fluid for counting whereas tissues were directly combusted before counting and faeces were lyophilised, homogenized and combusted prior to

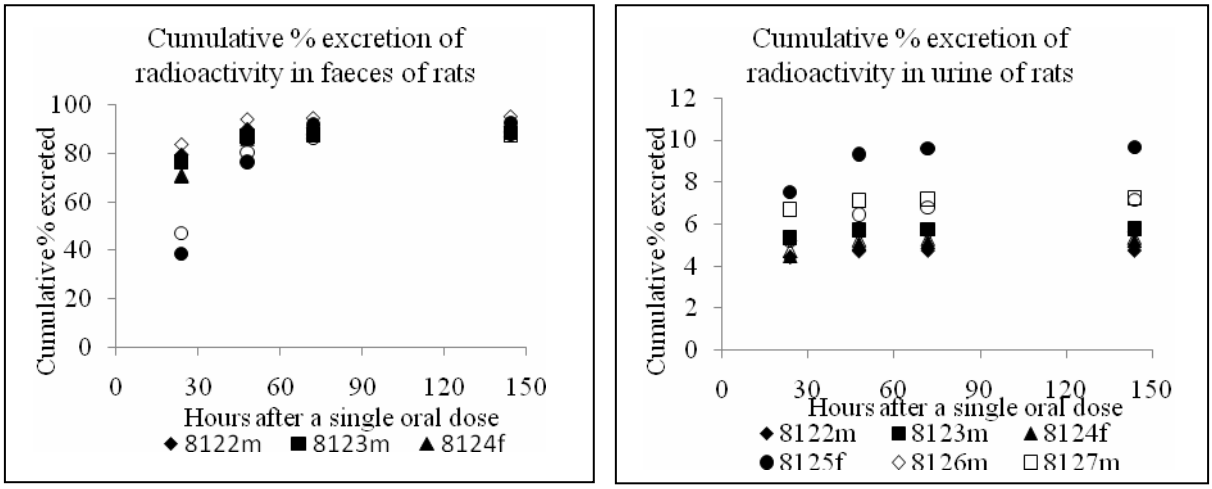
counting. Samples of faeces were extracted with methanol/water 80:20 and subjected to co-chromatography on TLC plates with reference standards.

Amounts of expired $^{14}CO_2$ were minimal (<0.05% of the administered dose). Radioactivity was primarily excreted in faeces and to a lesser and more variable extent in urine. Table 1 shows the cumulative percentage excretion of radioactivity of total dose administered in faeces and urine calculated over a time period of 144 hours (6 days). The results suggest that recovery was approximately 97% after 144 hours in this study. Individual data points are given in Figure 1.

Table 1: Cumulative percentage excretion of radioactivity in rats after a single oral dose of either 0.5 or 25 mg [^{14}C]-triclabendazole/kg of bw, relative to dose administered.

	Results obtained with the low dose			Results obtained with the high dose			Results of both dose levels combined		
	Faeces	Urine	Faeces plus urine	Faeces	Urine	Faeces plus urine	Faeces	Urine	Faeces plus urine
Parameter estimate	Percent of radioactivity recovered in 144 hours after a single oral dose								
Mean	90.9	6.1	97.0	90.1	6.3	96.5	90.5	6.2	96.7
St Dev	1.8	2.4	3.5	3.5	1.0	2.9	2.6	1.7	3.0
Min	88.4	4.2	94.2	87.8	5.3	95.0	87.8	4.2	94.2
Max	92.6	9.6	102.2	95.2	7.3	100.8	95.2	9.6	102.2

Figure 1: Cumulative percentage excretion of radioactivity in rats after a single oral dose of either 0.5 or 25 mg [^{14}C]-triclabendazole/kg of bw, relative to dose administered.

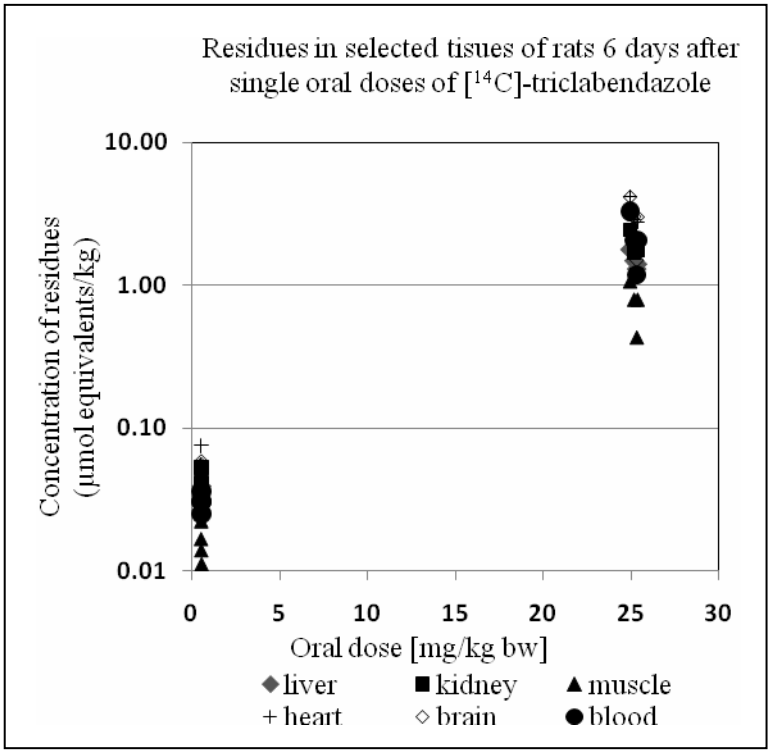


The code numbers in the legend refer to animal ID; m = male; f = female. The filled symbols indicate results obtained with the lower dose (approximately 0.5 mg/kg of bw); the open symbols indicate results obtained with the higher dose (approximately 25 mg/kg of bw).

The extracts of faeces contained some unchanged drug (7% of dose), but mainly the corresponding sulphoxide (24% of dose) and small amounts of the sulphone (2% of dose) metabolites. Approximately 27% of the radioactivity in faeces was not extracted with three sequential extractions with the methanol/water solvent. The dose had no significant influence on the qualitative metabolite pattern. The structure of the more polar metabolites in urine could not be determined in this study.
Residues in selected tissues were determined six days after dose administration. Residue concentrations found were highest in heart, brain and blood. The individual results for some selected

tissues (liver, kidney, muscle, heart, brain and blood) are given in Figure 2. Residues in fat were below the limit of detection (0.06 mg/kg), except in one sample obtained from a rat that had received the higher dose. The administered high and low doses differed by a factor of 47.4. The ratio of radioactivity found in the tissues (geometric mean) represented in Figure 2 was 40.7, 42.9, 47.8, 49.4, 57.9 and 67.2 for liver, kidney, muscle, heart, brain, and blood, respectively. An increase in dose had an over-proportional effect on residue distribution into certain tissues.

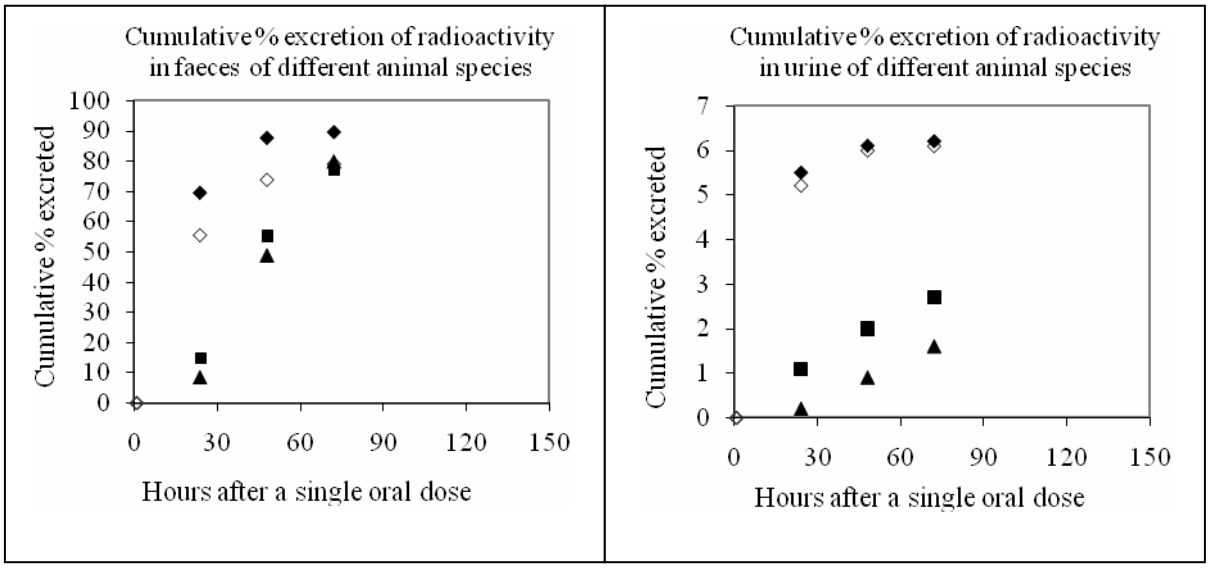
Figure 2: ^{14}C residues in tissues of rats six days after a single oral dose of approximately 25 mg [^{14}C]-triclabendazole/kg of bw.



Rats, sheep and goats

After a single oral dose of 10 mg/kg of bw to one sheep and to one goat and 0.5 or 25 mg/kg of bw in two rats (one male and one female), excretion of radioactivity was monitored for 72 hours in faeces and urine (Hamböck, 1983). The rates of excretion in faeces and urine of rats relative to the total dose administered were similar to those in the study of Muecke, (1981); however, they were lower for both routes in the female sheep and in the female goat at early time intervals. Excretion was slowest in the goat. The results obtained with the individual animals are shown in Figure 3.

Figure 3: Excretion data obtained in the study of Hamböck (1983).



Legend: Solid square = female sheep; solid triangle = female goat; open diamond = low dose male rat; solid diamond = high dose female rat

Metabolites were determined in samples of pooled faeces (0-72 hours in a sheep, a goat and a male rat; 0-48 hours in a female rat); the radioactivity in these samples corresponded to 76.7, 79.8, 90.0, and 87.6 % of the total dose in the sheep, goat, male rat and female rat, respectively. Some 50-72% of the radioactivity was extractable with methanol. Metabolites were identified by co-chromatography with reference standards on TLC plates. Structures were further confirmed by specific transformations using chemical reduction/oxidation reactions, mass spectrometry and nuclear magnetic resonance. Similarly, pooled urine samples were analysed. Metabolites in urine were generally more polar than metabolites in faeces. The least polar metabolite in urine was keto-triclabendazole.

Four major metabolites in addition to the parent drug were identified in faeces of all three species. In the sheep and goat, most of the excreted metabolites were unchanged parent drug, however, in rats, the sulphoxide was the major excreted metabolite (Table 2). The difference between the two ruminant species and rats was assumed to reflect differences in intestinal flora rather than differences in biotransformation pathways.

Table 2: Characterisation of radioactive substances extracted from pooled faeces.

Species	Rat		Sheep	Goat
Sex	male	female	female	female
Dose (mg/kg bw)	0.5	25	10	10
Identification of the radioactive zone on TLC plates	% of administered dose			
6-chloro-5-(2', 3'-dichlorophenoxy)-2-methylthio-1-*H*-benzoimidazole (parent drug) (CGA 89 317) $C_{14}H_9Cl_3N_2OS$; MW: 359.66	6	9	19	25
6-chloro-5-(2',3'-dichlorophenoxy)-2-methylsulfinyl-1-*H*-benzimidazole (sulphoxide) (CGA 110 752) $C_{14}H_9Cl_3N_2O_2S$; MW: 375.66	20	27	7	6
6-chloro-5-(2',3'-dichlorophenoxy)-2-methylsulfonyl-1-*H*-benzimidazole (sulphone) *plus minor unknowns* CGA 110 753 $C_{14}H_9Cl_3N_2O_3S$; MW: 391.66	3	3	2	2
5-chloro-6-(2',3'-dichlorophenoxy)-1,3-dihydro-2*H*-benzimidazol-2-one (keto-triclabendazole) (CGA 110 754) $C_{13}H_7Cl_3N_2O_2$; MW: 329.57	8	10	2	3
Minor unknowns plus 6-chloro-5-(2',3'-dichloro-4-hydroxyphenoxy)-2-methylthio-1-*H*-benzimidazole (hydroxy-triclabendazole) (CGA 161 944) $C_{14}H_9Cl_3N_2O_2S$; MW: 375.66	11	12	13	9
Unknowns	9	13	6	6
Non-extractable	32	16	27	29

The elimination of triclabendazole and its metabolites was also investigated in a bile duct-cannulated male rat receiving 4.55 mg/kg as a single oral dose. In this study, 34% of the dose was excreted with the bile. Comparison of the results obtained with bile duct-cannulated and non-cannulated rats found that a significant proportion of the absorbed dose was eliminated in bile and only a small proportion of the radioactivity in faeces is unabsorbed triclabendazole. The biliary metabolites were not further characterised; however, the investigators noted that they were not acid-labile.

Rats

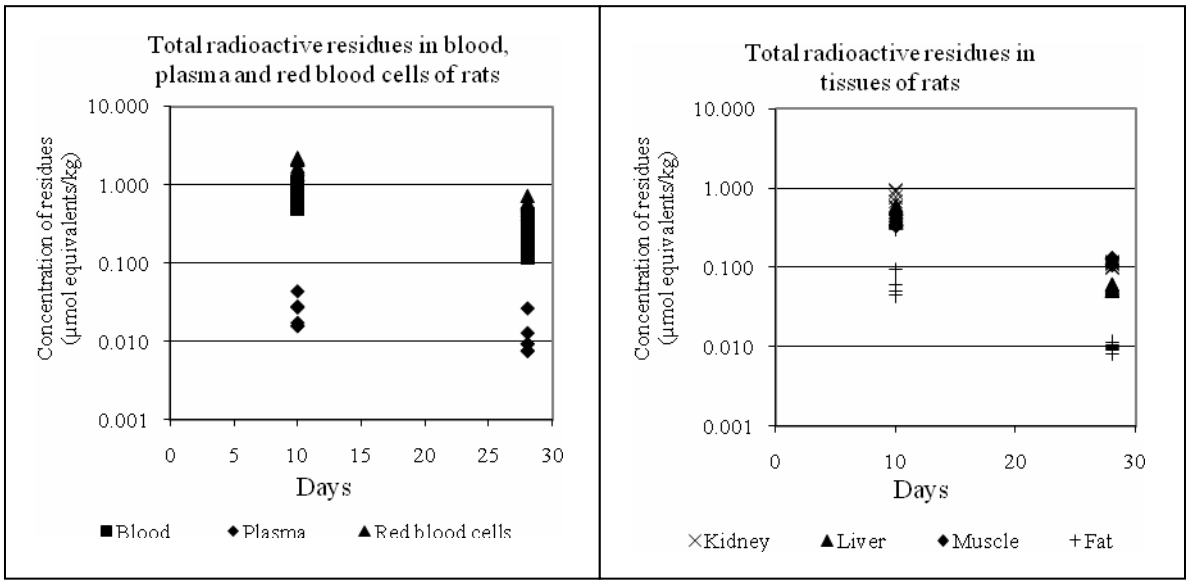
Excretion balance and tissue distribution studies (Hardwick, 2004a) were conducted in twelve Sprague Dawley rats dosed orally by gavage at a nominal dose rate of 12 mg (range 10.32-12.14 mg) triclabendazole per kg of bw. Triclabendazole was labelled in the benzene ring of the benzimidazole moiety (specific activity 13.9 MBq/mg). Urine and faeces and expired air were collected from six rats for up to 10 days. At 10 days after dose administration these rats were sacrificed and samples of blood, liver, kidney, muscle and fat were collected. At 28 days after dosing, the remaining six rats were sacrificed and the same types of samples obtained. Radioactivity was determined in blood, plasma, red blood cells, urine, faeces, expired air, cage washes, liver, kidney, muscle and fat. The recovery after 10 days from faeces and urine was variable (Table 3) ranging from 88.2 to 127.7 % of the administered dose per animal, suggesting methodological uncertainties. None of the radio-labelled residues showed similar chromatographic properties to the supplied reference standards; however, co-chromatography showed that all the residues present in cow tissues were also present in rat tissues.

Table 3: Total recovery of radioactivity 0-10 days following a single oral dose of 12 mg [^{14}C]-triclabendazole/kg of bw to male rats.

Animal ID:	101M	102M	103M	104M	105M	106M
Dose (mg/kg)	10.6	10.3	12.1	10.4	11.1	10.3
Matrix	Recovery (% of dose)					
Urine	7.7	10.0	6.8	10.1	7.8	3.9
Faeces	86.5	78.2	98.1	88.9	119.8	106.6
Cage Wash	6.2	3.3	2.0	2.3	1.4	0.3
Cage Debris	<LOQ	0.009	0.010	0.003	0.025	<LOQ
Expired Air	0.007	0.003	0.006	0.008	0.005	<LOQ
Tissues	0.16	0.19	0.19	0.28	0.17	0.17

Residues in tissues after 10 and 28 days, respectively, are shown in Figure 4. Concentrations of residues were highest in erythrocytes and lowest in fat. The rate of depletion between the two time points was highest in fat, followed by liver and kidney and the lowest in muscle and the constituents of blood. Approximately 80% of the residues in liver were non-extractable. The extractable residues showed a wide range of polarities. Alkaline hydrolysis of the tissues followed by acidification increased the extraction efficiency. The reference standards were unaffected by alkaline hydrolysis, with the exception of triclabendazole which hydrolysed to a less polar compound. The authors reported that it is probable that the triclabendazole moiety in the residues extracted after alkaline hydrolysis was intact, although covalently bound (via the sulphur atom) to a cellular component that had been cleaved by hydrolysis. At least seven bound residues were present in alkaline tissue extracts.

Figure 4: Residue depletion in selected tissues of rats dosed orally at a nominal dose of 12 mg [^{14}C]-triclabendazole/kg of bw.



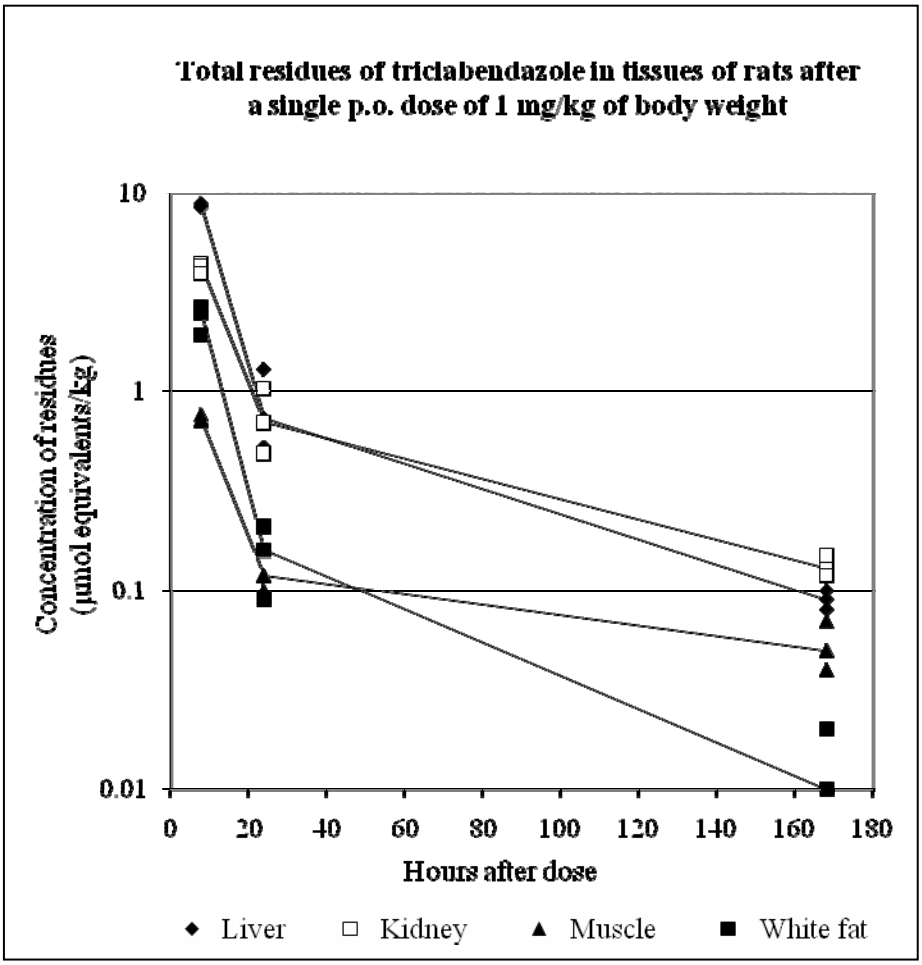
The results of studies into the extractability following NaOH hydrolysis are shown in Table 4 and indicate that 2M NaOH was equally efficient in solubilising parts of the residues in red blood cells, liver and kidney.

Table 4: Partitioning of radioactive residues between dichloromethane and water following treatment with sodium hydroxide (NaOH).

Tissue	Treatment	Dichloromethane Extractable (%)	Remaining in aqueous phase (%)
Red blood cells	2M NaOH	80	12
Liver	0.2 M NaOH	49	39
Liver	2M NaOH	78	25
Muscle	0.2 M NaOH	65	26
Kidney	2M NaOH	75	14

The distribution of radioactivity in blood, plasma and 22 organs and tissues of rats was determined after single i.v. and p.o. administrations and multiple p.o. dosing of 1 mg [^{14}C]-triclabendazole/kg of bw. At 8 hours after an oral dose, residue concentrations were highest in liver, followed by kidney, heart, white fat and lung, brain and muscle. The kinetics of depletion were biphasic with overall rates decreasing in the order of white fat, liver, lung and kidney, muscle, heart and brain. Concentrations in most tissues at 168 hours after dosing were still slightly lower after p.o. dosing compared to i.v. administration. Figure 5 shows some examples of depletion kinetics (the lines connect the median values of three data points of the same tissue type). Once daily dosing with 1 mg [^{14}C]-triclabendazole/kg of bw for 10 days resulted in significant accumulation of residues in all tissues except plasma. The accumulation was most significant in brain and heart.

Figure 5: Depletion of radioactive residues after a single oral dose of 1 mg [^{14}C]-triclabendazole/kg of bw to rats.



Excretion of total radioactivity in urine and faeces of some rats and dogs was determined at some of the same dose levels used for establishing the kinetics in blood and plasma. Excretion was not complete in rats and even less complete in dogs after 168 hours (see Table 5). The fraction of the dose that was excreted in urine was smaller in dogs than in rats and decreased further with increasing oral doses in both species.

Table 5: Cumulative excretion of total radioactivity in urine and faeces of rats and dogs

Dose (mg/kg bw)	Route	Rats						Dogs	
		RA16	RA17	RA 18	RA4	RA5	RA6	1014	1016
		Cumulative excretion (0-168 hrs) in urine and faeces (%)							
0.5	i.v.							83.3	77.2
0.5	p.o.							58.9	51.8
1	i.v.	89.7	88.3	90.4					
1	p.o.				92.7	95.1	94.3		
5	p.o.							68.8	
40	p.o.								89.7

The pharmacokinetics of [^{14}C]-triclabendazole (specific radioactivity of 13.9 MBq/mg and radiochemical purity of 99.7%) was studied following p.o. and i.v. administration to 6-12 weeks old male Sprague Dawley rats weighing 0.27 - 0.37 kg (Needham, 2004a). Lyophilised tissue test material in the study was obtained from cattle treated with [^{14}C]-triclabendazole of specific radioactivity of 6.585 MBq/mg (Needham, 2004b). The design of the study is shown in Table 6.

Table 6: Design of the Needham (2004a) Sprague Dawley rat study

Group	Route and method of administration	Test material	Dose (mg/kg bw)	Number of animals
A	Oral gavage	[^{14}C]-triclabendazole	0.25 $\pm$ 0.001	6
B	Intravenous	[^{14}C]-triclabendazole	0.30 $\pm$ 0.006	6
C	Dietary	[^{14}C]-triclabendazole	0.24 mg $\pm$ 0.034	6
D		lyophilised muscle [1]	0.0013 – 0.0059	6
E		lyophilised liver	0.25 - 3.46 µg	6
F		lyophilised kidney	0.00022 – 0.0023	3
G		lyophilised muscle	0.0035 – 0.0079	5
H		lyophilised liver [2]		5
I	Oral gavage	lyophilised liver	0.0015	3

[1] [^{14}C]-triclabendazole equivalents

[2] Rats did not eat the dose and were removed from the study and allowed to recover for one week. Three of the rats were then dosed orally by gavage with an aqueous suspension of lyophilised liver (Group I).

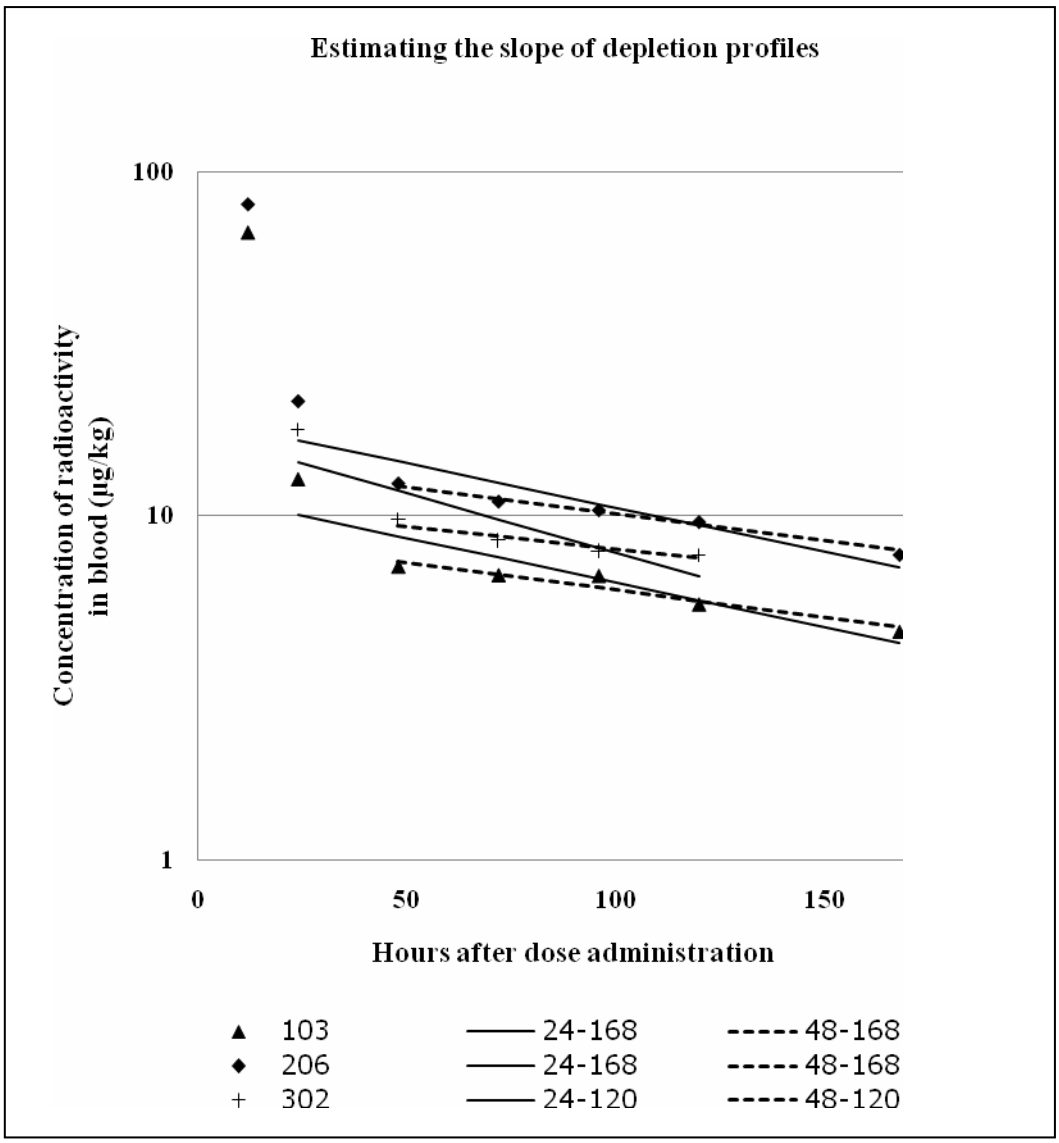
Rats receiving lyophilised tissues were allowed to eat the diet for 4 h before it was removed, weighed, and replaced with normal diet. Blood samples (150 µL) were taken 1, 2, 3, 4, 6, 9, 12, 24, 48, 72, 96 and 120 hours after initial exposure to the diet containing [^{14}C]-triclabendazole (Group C) or lyophilised tissues with incurred residues (Groups D-H). Blood samples were also collected 30 minutes (Group A) and 20 minutes (Group B) after dosing with [^{14}C]-triclabendazole, and 168 hours (Groups C-F at necropsy) after dietary exposure to lyophilised tissues. Liver, kidney and muscle were taken from the rats in Groups A and C-F at necropsy. With Group G, blood samples were taken only from the three animals that consumed the largest quantity of tissue.

The kinetics of the concentration of radioactivity were studied for 168 hours in animals of Groups A and B and for 120 hours in Groups C, G, and I. Detectable concentrations of radioactivity in blood were measured by liquid scintillation counting for all animals following oral, intravenous or dietary

dosing with [^{14}C]-triclabendazole (Group A-C). Data received from Groups D-F were insufficient to determine the pharmacokinetic parameters of the absorbed radioactivity in these animals. Using accelerated mass spectrometry (AMS), it was also possible to determine the levels of radioactivity in the groups that had received lyophilised tissues.

The estimation of bioavailability of the radioactive marker is based on calculations of the $AUC_{0\text{-infinity}}$. These calculations showed that the terminal elimination was not yet complete at 120-168 hours after dosing, the last time point at which blood samples were taken. Figure 6 highlights a problem when estimating the slope of the depletion profiles. The study authors consistently used the results obtained 24 hours after dosing for calculating terminal half-life; however, it is evident from the three examples given in Figure 6 that the concentrations measured at 24 h are dependent on earlier phases of the disposition kinetics.

Figure 6: Estimation of the slope of depletion profiles for calculating terminal half-life and $AUC_{t\text{-infinity}}$ in the Needham (2004a) study.



The graph shows (solid triangle symbols) the last 6 data points of the kinetics obtained with animal 103 (dosed by gavage with 0.25 mg/kg of bw of labelled triclabendazole). The solid line shows the basis for the calculation of the terminal half-life by the authors of the study. The dotted line shows the

difference if the calculation is based on the last five data points only. The difference is significant. The same is true regarding the results obtained with animal 206 (dosed i.v. with 0.30 mg/kg of bw) (shown as solid diamond symbols). The influence on the calculated $AUC_{t\text{-infinity}}$ is significant due to the steeper slopes, the terminal half-lives calculated by the authors are typically shorter and the values of AUC smaller compared with the results of a more adequate calculation. However, since each pair of lines run in parallel the influence on the ratios of the AUCs is minimal and "correct" estimates of the blood bioavailability of doses given by gavage are obtained. The situation is different if one looks at the evaluation of the results obtained with animal 302 (exposed to 0.26 mg/kg of bw in the diet). In this case (cross symbols), the "incorrectly" calculated lines no longer run in parallel, however, the "correctly" calculated lines still do.

The results of the whole experiment were re-calculated in this way. Graphs of all depletion curves were prepared and the data points primarily influenced by the terminal elimination were selected. Using these points the terminal half-lives and the $AUC_{t\text{-infinity}}$ were recalculated and the following results were obtained. All terminal half-lives calculated in this way were longer than those reported by the authors and all values for the $AUC_{t\text{-infinity}}$ were higher. This had no significant influence on the estimated bioavailability when the animals were dosed by gavage; however, in the case of dietary exposure to incurred residues, the calculated bioavailability was increased. Table 7 compares the results of the re-calculation with those obtained by the authors.

Table 7: Results of recalculation of selected results of the Needham (2004) study.

Treatment group	Mean Bioavailability		Mean Terminal Half-life (hrs)	
	Calculated by the authors	Re-calculated	Calculated by the authors	Re-calculated
A	0.715	0.694	147.7	197.4
C	0.676	0.913	91.4	289.4
G	0.064	0.086	90.7	203.7
I	0.098	0.094	135.9	164.9

The re-calculated terminal half-lives are significantly longer than those reported by the authors. The effects on calculated bioavailability are negligible for the experiments with gavage administration (Groups A and I), however they are significant for the dietary exposure (Groups C and G). In general, terminal half-lives are longer than estimated by the authors. In terms of dietary exposure, a weakness of the study design was the absence of sampling points later than 120 hours after exposure. This was also problematic for the re-calculation insofar as frequently too few data points were available for a fully adequate estimation. The main finding of the authors remains unchallenged, namely the bioavailability of residues from incurred tissues (animals sacrificed 28 days after treatment) is low.

These data demonstrated that the absolute bioavailability of [^{14}C]-triclabendazole was approximately 70% when given by gavage to rats. By comparison, the absolute bioavailability of incurred residues administered by gavage to rats was 9.2% for liver, which was higher than for other tissues. Therefore the calculated bioavailability of incurred liver residues in cattle was 13% (9.2/70 x 100) relative to the oral gavage treatment.

Rabbits

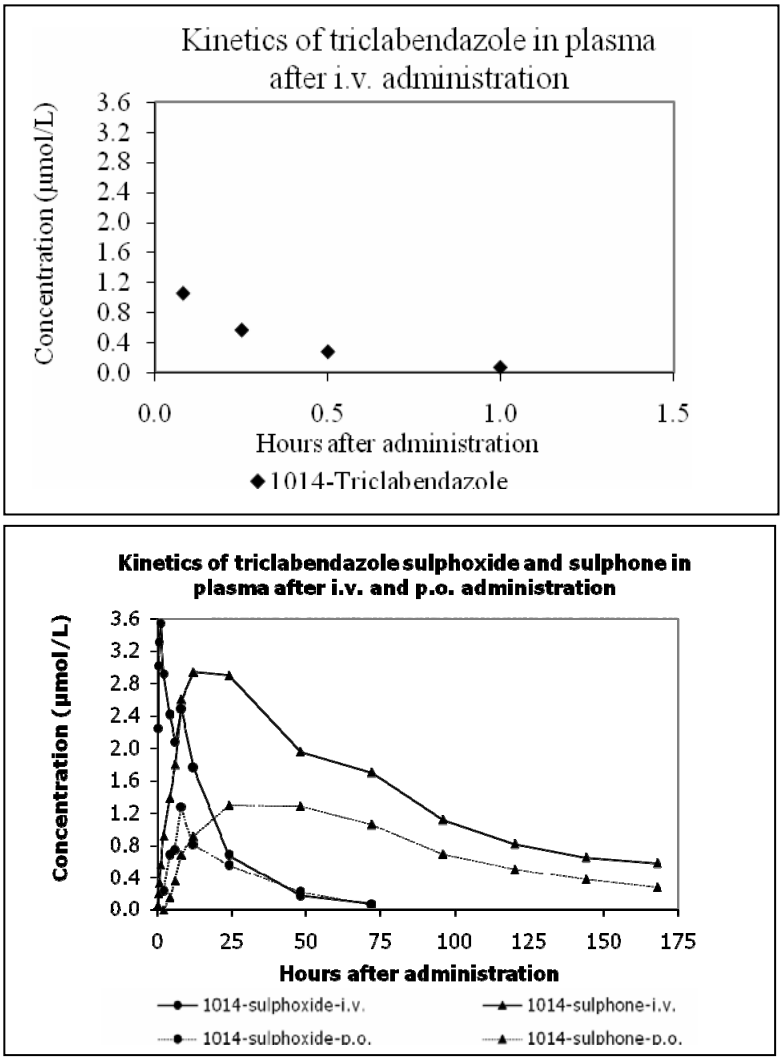
[^{14}C]-labelled triclabendazole was administered i.v. and p.o. to two female Chinchilla rabbits (Wiegand, et al., 1991a). The animals ranged from 2.7 to 4.3 kg over the duration of the study. The doses were administered at intervals of at least 4 weeks, first with an i.v. dose of 3 mg/kg of bw, then with oral doses of 3 mg/kg and 26 mg/kg of bw. The concentration of total radioactivity in blood and plasma, and excretion with urine and faeces, were measured. The absorption of triclabendazole from the gastrointestinal tract was complete irrespective of the dose rate. Radioactive substances in blood demonstrated a biphasic decay in plasma. Most of the radioactivity was cleared from the circulation

within 72 hours, predominantly in bile. However, approximately 17-20% of the radioactivity had not been excreted 7 days after dosing. In addition, plasma concentrations of unchanged triclabendazole, and of its sulphoxide and sulphone metabolites, were determined (Wiegand, et al., 1991b). At 5 minutes after i.v. injection, the concentration of triclabendazole sulphoxide was higher than that of triclabendazole. Following oral dosing, no triclabendazole was detected in plasma. The formation of the sulphone was slower than for the sulphoxide. These two metabolites represented the total radioactivity measured in plasma for the first 8 hours after dosing.

Dogs and Rats

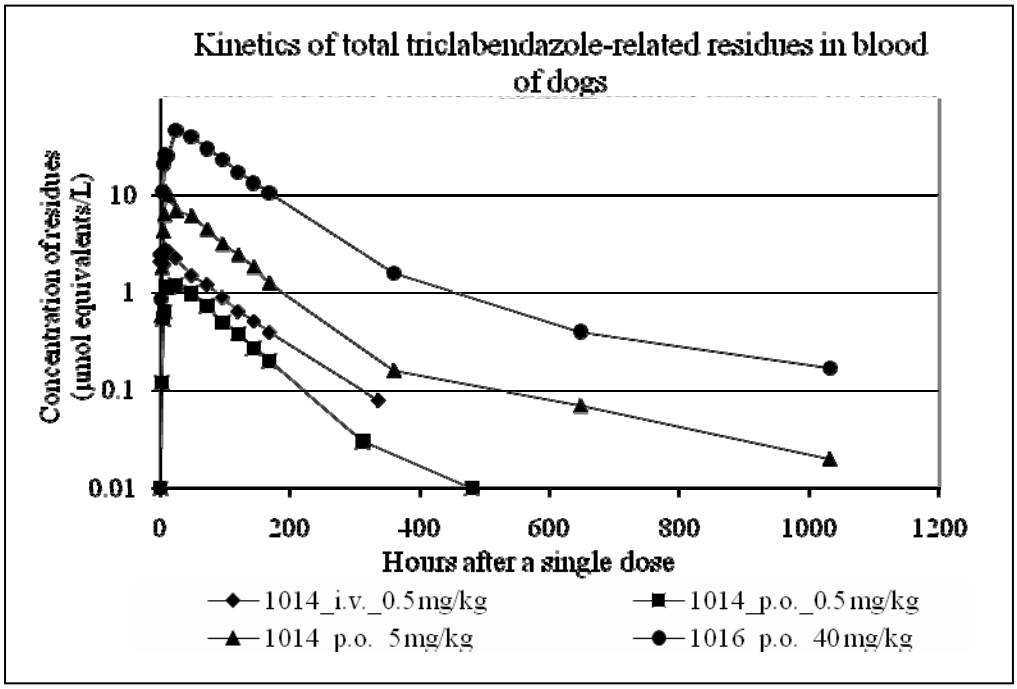
A large study in dogs and rats was conducted that investigated the absorption, distribution and excretion of [^{14}C]-triclabendazole (Schütz, et al., 1991). The concentrations of triclabendazole and its sulphoxide and sulphone metabolites in plasma and urine of dogs and rats, following i.v. and p.o. administration of [^{14}C]-labelled triclabendazole, were reported. The plasma kinetics of the parent drug after i.v. administration of 0.5 mg/kg of bw to one of two beagle dogs, and of the sulphoxide and sulphone metabolites after p.o. administration of the same dose to the same dog, are shown in Figure 7. Triclabendazole was rapidly converted to its sulphoxide and sulphone metabolites. After i.v. administration, the parent drug rapidly disappeared and the concentration of triclabendazole sulphoxide immediately increased. No unchanged drug could be detected beyond 1 hour after injection. After oral administration of 0.5 and 5 mg/kg doses, no triclabendazole was detected in plasma; the sulphone was slowly formed and eliminated. The renal elimination of triclabendazole was negligible in dogs.

Figure 7: Plasma kinetics of triclabendazole and its major metabolites after a single i.v. or oral dose of 0.5 mg/kg of body weight to a beagle dog (animal 1014).



The kinetics of total radioactivity in plasma and blood were also determined after i.v. and p.o. administration of 0.5 and 5 mg/kg of bw and 40 mg/kg of bw p.o. in dogs, and after 1 mg/kg of bw i.v. and p.o. and 10 mg/kg and 80 mg/kg of bw p.o. in rats (Schütz, et al., 1996). Figure 8 shows selected results of blood analyses for total radioactivity obtained with two dogs (animals 1014 and 1016).

Figure 8: Kinetics of total radioactive residue in blood of dogs dosed with [^{14}C]-triclabendazole



The concentrations of total radioactivity in plasma were initially higher than those determined in whole blood. The ratio of the concentrations in blood to plasma was approximately 0.59 at the lowest dose level and was the same for the i.v. and p.o. routes of administration. The ratio decreased with increasing oral doses, but was constant over several days for a given dose. The sum of the concentrations of the sulphoxide and the sulphone in plasma (given in Figure 7) correlates well with the concentrations of total radioactive residue.

The ratio of the concentrations in blood to plasma was in the order of >0.7 after an i.v. dose of 1 mg/kg of bw and >0.6 after an oral dose of 1mg/kg of bw in rats. However, the ratio increased over time and was >1 after 24 hours. After two days, the ratio was 3.2-5.5 (n=3) for the i.v. treatment and 7-8 (n=3) after p.o. treatment.

The kinetic profiles of the radioactive residues were also used to determine the area under the concentration-time curves. The results are summarised in Table 8. Calculated plasma bioavailability was practically 100% in rats at an oral dose of 1 mg/kg bw (n=3). It decreased slightly up to a 10-fold dose level and decreased significantly further up to an 80-fold dose level. The calculated plasma bioavailability in dogs was approximately 37.7 - 55.6% on the basis of the $AUC_{0\text{-infinity}}$ (n=2), 43.8 % at a dose of 0.5 mg/kg bw (n=1) and 26.8% (n=1) at a dose of 40 mg/kg of bw. Each rat was tested only at one dose level in this experiment; however, each of the two dogs of the experiment was tested at three dose rates.

Table 8: Specific AUC (dose corrected; time from 0 to 168 hours after treatment) of total radioactive residues in blood and plasma of dogs and rats.

Dose (mg/ kg bw)	Route	Matrix	Dogs		Rats									
			1014	1016	RA13	RA14	RA15	RA1	RA2	RA3	RA101	RA102	RA105	RA106
			Specific AUC [µmoles x h/(L x mg/kg)]											
0.5	i.v.	Blood	405	503										
		Plasma	686	881										
	p.o.	Blood	215	174										
		Plasma	353	303										
1	i.v.	Blood			49	61	55							
		Plasma			55	66	61							
	p.o.	Blood						53	61	55				
		Plasma						58	66	57				
5	p.o.	Blood	141											
		Plasma	252											
10	p.o.	Blood									46	37		
		Plasma												
40	p.o.	Blood		106										
		Plasma		213										
80	p.o.	Blood											26	27
		Plasma												

Food Producing Animals

Cattle

A dose of 12 mg of [^{14}C]-triclabendazole (specific activity of 82.6 µCi/mg and radiochemical purity of 95.7%)/kg of bw was administered by oral capsule to one Angus heifer (animal 159) and one Hereford heifer (animal 156), both approximately 7 months of age and weighing 177 kg and 160 kg, respectively (Downs, et al., 1991). Animal 159 was sacrificed at 28 days and animal 156 was sacrificed at 42 days after dosing and tissue samples were collected for combustion analysis to determine [^{14}C]. The results are summarised in Table 9.

Table 9: Total [^{14}C] residues in tissues from treated beef heifers.

Tissue	Beef Heifers	
	Animal 159 (sacrificed 28 days after dosing)	Animal 156 (sacrificed 42 days after dosing)
	Residues (mg/kg equivalents*	
Liver	0.24 ± 0.013	0.09 ± 0.009
Kidney	0.11 ±0.016	0.07 ± 0.011
Muscle (composite)	0.13 ± 0.017	0.10 ± 0.007
Fat (composite)	0.01 ± 0.003	<0.01 ± 0.001

* Data are mean ± standard deviation

Tissue samples derived from the Angus heifer (animal 159) sacrificed 28 days after drug administration in the study by Downs, et al. (1991) were sequentially extracted on three occasions each with methanol and ethyl acetate, and the extracts were radioassayed (Krautter, 1992). Low extraction efficiencies did not allow for the incurred tissue residues to be characterised by chromatography.

[^{14}C]-triclabendazole (specific radioactivity of 5.96 MBq/mg and radiochemical purity of 95.7%) was administered by gavage as a single dose of 12 mg/kg of bw to one female Aberdeen Angus and one male Friesian/Limousin cross ruminating calf weighing 63 kg and 96 kg, respectively, at the time of dosing (Ferguson, 1994a). Faecal and urinary excretion for 0-168 hours post-dosing accounted for 76% and 2.2% of the administered radioactivity, respectively. Plasma protein binding exceeded 99% in all samples. Both animals were sacrificed at 28 days after dosing. Radioactivity was determined in liver, kidney, tenderloin muscle, hindquarter muscle, forequarter muscle, perirenal fat, subcutaneous fat, plasma and red cells. Radioactivity was present in all tissues sampled with highest levels in liver, followed by muscle and kidney with the lowest levels present in fat.

In a separate study by Dieterle and Kissling (1995), the tissue samples from the above study (Ferguson, 1994a) were analysed in the context of a validation study for method REM 15/83. Extractability with dichloromethane was 102% (muscle), 64% (liver), 82% (kidney), and 97% (perirenal fat) for cattle. The accountability (not corrected for procedural recoveries) of method REM 15/83 with UV detection was 34% (muscle), 14% (liver), and 22% (kidney) of total residues for cattle. These results are discussed later in relation to dietary intake and are summarised in Table 21.

A study by Thanei (1995a) was a continuation of the Ferguson (1994a) study. Specifically, the metabolite pattern in extracts of urine and faeces derived from cattle was quantitatively determined and the individual metabolites were characterised. Approximately 2% of the administered dose was eliminated in urine collected up to 168 hours after dosing. Four metabolites of triclabendazole but no parent *per se* were detected in urine. By comparison, approximately 76% of the administered dose was eliminated in faeces collected up to 168 hours after dose administration. The major metabolites in faeces were triclabendazole, its sulphoxide and sulphone, and 2,3-dichloro-4-(6-chloro-2-methylsulfanyl-3H-benzoimidazol-5-yloxy)-phenol and its sulphone.

A male ruminating Holstein Friesian calf aged 9 weeks and weighing 91 kg was administered a single oral dose by capsule of 12 mg [^{14}C]-triclabendazole (specific radioactivity of 13.9 MBq/mg and radiochemical purity of 99.5%)/kg of bw (Needham, 2004b). Urine and faeces collected at 24 h intervals until 10 days after dosing accounted for 78.2% and 3.4% of the administered radioactivity, respectively. The calf was sacrificed at 28 days after dosing and the tissue distribution of radioactivity was determined (Table 10).

Table 10: Concentration of radioactive residue in the tissues of a calf at 28 days after administering a single oral dose of 12 mg [^{14}C]-triclabendazole/kg of bw.

Tissue	Concentration of radioactivity as µg equivalents of triclabendazole/kg of tissue
Liver	283.3
Kidney	163.3
Muscle	209.1
Fat	25.8
Blood	70.4
Red blood cells	63.1
Plasma	51.1

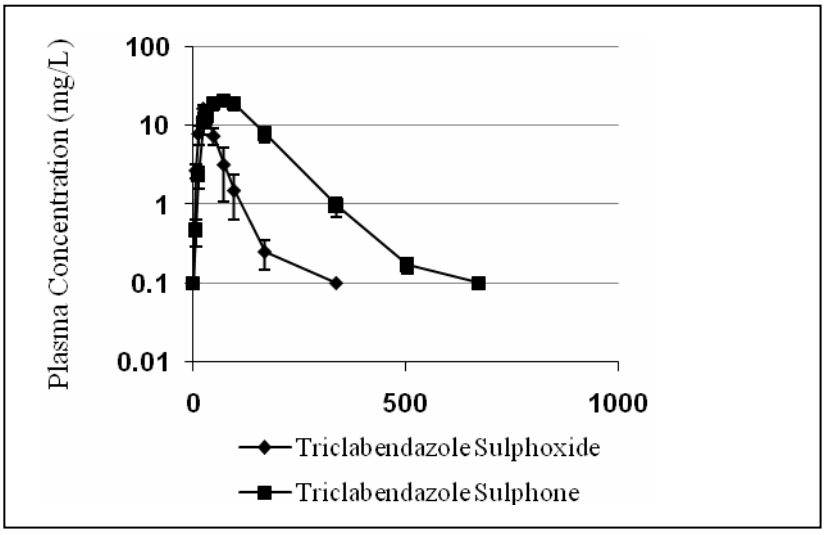
A fractionation study (Needham, 2004b) showed that 92-98.6% of the radioactivity in liver and muscle was associated with either lipid or protein. Changing the pH of the extracting solvent from acidic to basic did not release the tissue residues, suggesting that they are covalently bound to macromolecules in cells. The triclabendazole-derived moiety of the residue was not released as a free metabolite with either alkaline hydrolysis or protease digestion whereas oxidation cleaved the ketone CGA-110 754 (keto-triclabendazole). Other metabolites, derived from phenolic metabolites of triclabendazole (eg, CGA-161 944 and CGA-183 196), could be released from the extract but were not carried through the clean-up process of the residue analysis method. The extraction of

radiolabelled incurred residues was determined for a range of solvents (Needham, 2004b). The most efficient extraction involved alkaline hydrolysis of the tissue with 2M NaOH. Under these conditions, 70–85% of the total radioactive residues was extractable with dichloromethane; however, the resultant extracts were difficult to analyse by HPLC, and no data were obtained from HPLC/MS.

A validated residue method with a limit of quantification of 0.03 mg/kg (expressed as triclabendazole equivalents for each bovine tissue) accounted for 26.5% (liver), 29.4% (kidney) and 34.9% (muscle) of the total radioactivity present in these tissues (Needham, 2004b). In plasma, the presence of triclabendazole-protein conjugates resulted in 90% of the radioactivity precipitating with the protein fraction. Storage stability of incurred residues in samples of cattle tissues stored frozen for 184 days (muscle) and 194 days (liver and kidney) was investigated (Needham, 2004b). Residues in muscle and kidney were stable during storage whereas with bovine liver, the mean concentration of triclabendazole after 6 months frozen storage declined to 72% of the initial concentration.

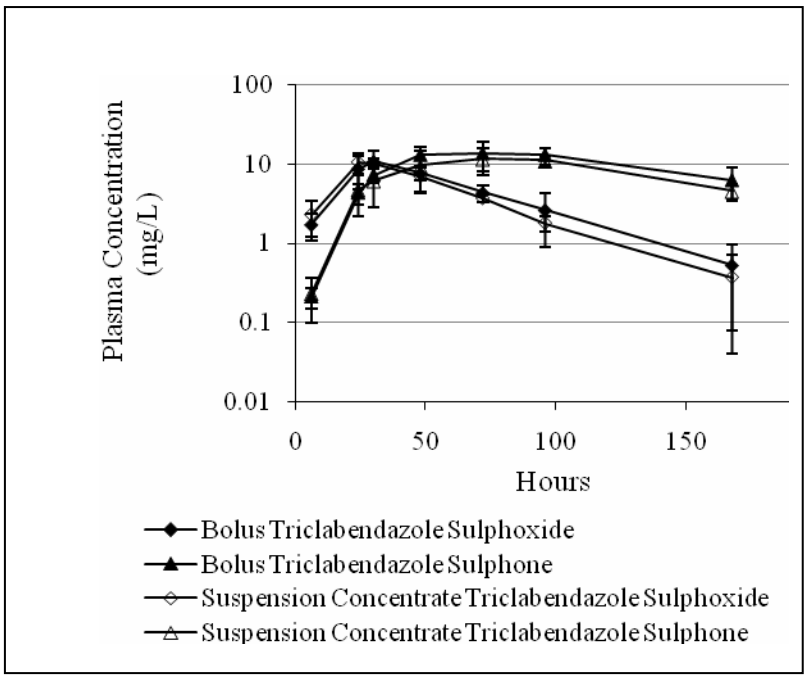
Four calves weighing 165-196 kg were administered an oral dose of 12 mg of triclabendazole (as a 10% w/v suspension)/kg of bw (Bull, et al., 1986a). Plasma samples were taken and analysed by HPLC for triclabendazole sulphoxide and triclabendazole sulphone. The semi-logarithmic plots of metabolite plasma concentration *versus* time are shown in Figure 9. The maximum plasma concentrations of the sulphoxide and the sulphone occurred at 24 h and 72 h, respectively.

Figure 9: Plasma concentration *versus* time profiles for triclabendazole sulphoxide and triclabendazole sulphone in cattle following an oral dose of 12 mg triclabendazole/kg of bw.



Ten 9 month-old Hereford-crossed calves weighing 192-238 kg were dosed with 12 mg triclabendazole/kg of bw as either a bolus (n=5) or 10% w/v suspension (n=5)(Bull et al, 1990). Animals receiving boluses were dosed to the nearest half bolus and the precise treatment rate was then calculated. Plasma samples were collected and analysed for triclabendazole sulphoxide and triclabendazole sulphone. The semi-logarithmic plots of metabolite plasma concentration *versus* time are shown in Figure 10. The bioavailability of triclabendazole was similar when administered to cattle by bolus and liquid suspension.

Figure 10: Plasma concentration *versus* time profiles for triclabendazole sulphoxide and triclabendazole sulphone in calves following an oral dose of 12 mg triclabendazole/kg of bw by bolus or 10% w/v suspension concentrate.



A study was conducted in cattle (Bull, et al., 1986b) which was similar to the sheep study conducted by Strong, et al.(1983). Six Friesian bulls, 10 months of age and weighing 186-236 kg, were assigned to one of two groups. Group 1 (n=3) were dosed i.v. with 12 mg triclabendazole (as a 10% w/v suspension)/kg of bw. Group 2 was dosed i.v. with 12 mg triclabendazole sulphoxide (as a 10% w/v suspension)/kg of bw. All animals in Groups 1 and 2 displayed adverse clinical signs after i.v. administration and one animal in Group 2 died. Plasma samples were collected for analysis by HPLC.

Semi-logarithmic plots of plasma metabolite concentrations *versus* time for the two groups are shown in Figures 11 and 12. With Group 1, the average maximum concentration of triclabendazole sulphoxide of 30.1 mg/l was observed approximately 4 hours after dosing and the maximum concentration of triclabendazole sulphone of 23.9 mg/l was observed approximately 32 hours after dosing. Plasma concentrations of triclabendazole were <0.1mg/l in 2 of the 3 animals by 12 hours after dosing. With Group 2, the average maximum concentration of triclabendazole sulphoxide of 159 mg/l was observed in the first blood sample taken at 2 minutes after dosing and the average maximum concentration of triclabendazole sulphone of 41.3 mg/l was observed at 32 hours after dosing.

Figure 11: Plasma concentration of triclabendazole sulphoxide and triclabendazole sulphone *versus* time after i.v. administration of 12 mg triclabendazole/kg of bw to cattle.

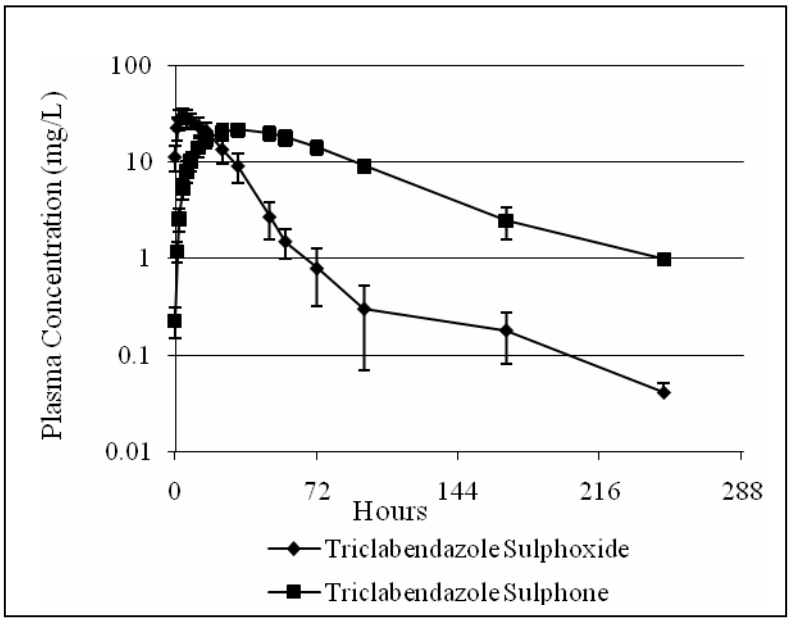
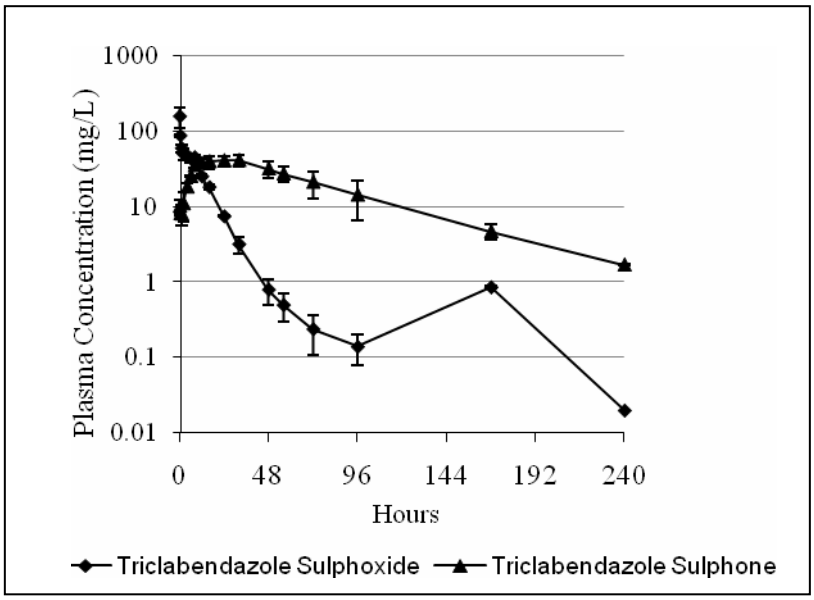


Figure 12: Plasma concentration of triclabendazole sulphoxide and triclabendazole sulphone *versus* time after i.v. administration of 12 mg triclabendazole sulphoxide/kg of bw to cattle.



In studies to determine whether the co-administration of triclabendazole and levamisole altered the pharmacokinetic behaviour of either compound, 21 calves were assigned to three groups (each n=7) (Strong, et al., 1987). Group 1 was dosed with 12 mg triclabendazole/kg of bw; Group 2 was dosed with 7.5 mg levamisole hydrochloride/kg of bw and 12 mg triclabendazole/kg of bw; and Group 3 was dosed with 7.5 mg levamisole hydrochloride/kg of bw. Plasma samples were collected and analysed for triclabendazole sulphoxide and triclabendazole sulphone and/or levamisole hydrochloride. Pharmacokinetic parameters for triclabendazole sulphoxide, triclabendazole sulphone and levamisole were calculated. The data demonstrated that co-administration of triclabendazole and levamisole did not significantly alter the pharmacokinetics of either compound in cattle.

Sheep

A comparison of the kinetic parameters of triclabendazole sulphoxide (CGA-110 752) and triclabendazole sulphone (CGA-110 753) in plasma following i.v. administration of triclabendazole (CGA-89 317) to sheep and cattle is shown in Table 11.

Table 11: Kinetic parameters of triclabendazole sulphoxide and triclabendazole sulphone in plasma following i.v. administration of triclabendazole to sheep and cattle.

Animal	Dose of CGA-89 317	Plasma profile	AUC (mg/L.h)	C_{max} (mg/L)	T_{max} (h)	$t_{1/2}$ (h)
Sheep	10 mg /kg of bw	CGA-110 752 CGA-110 753	651 596	42 13	42 13	14 27
Cattle	12 mg/kg of bw	CGA-110 752 CGA-110 753	795 2043	34 24	34 24	13 40

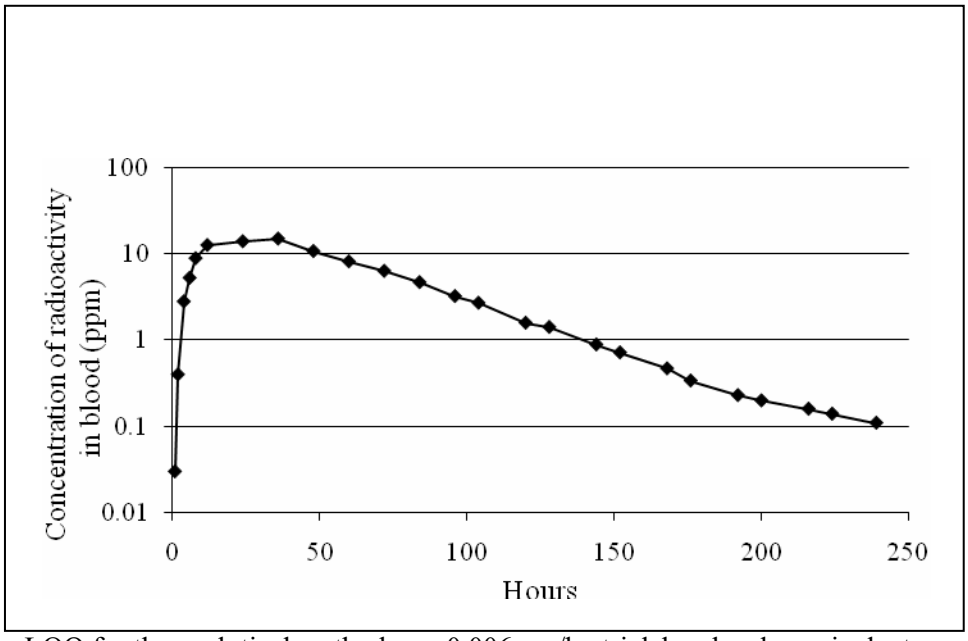
[^{14}C]–CGA-89 317 (specific radioactivity of 1.129 MBq/mg and radiochemical purity of 99%) was administered orally in gelatine capsules at a rate of 10.5 mg/kg to a 4 months old female sheep (Swiss White Alp X Ile de France breed), that weighed 28.5 kg (Hamböck and Strittmatter, 1982). Blood samples were collected and radioactivity in the samples was determined. The semi-logarithmic plot of radioactivity concentration in blood *versus* time is shown in Figure 13. The excretion of radioactivity in urine and faeces was measured every 24 hours to 10 days post-dosing. Faecal and urinary excretion accounted for 100.9% and 3.5% of the administered dose, respectively. The faecal extract (0-72 hours) contained unchanged triclabendazole (19.3% of the administered dose), triclabendazole sulphoxide (6.6%), triclabendazole sulphone (2.2%) and some unknown metabolites. The urine contained only polar fractions. The sheep was sacrificed 10 days after dosing and the tissue distribution of radioactivity investigated. The results are shown in Table 12.

Table 12: Radioactivity in selected tissues and organs of a sheep 10 days after oral administration of 10.5mg [^{14}C]-triclabendazole/kg of bw.

Tissue	mg/kg triclabendazole equivalents	Tissue	mg/kg triclabendazole equivalents
Blood	0.11	Rumen - wall	0.21
Liver	1.84	content	0.02
Gall bladder	0.40	Intestine- wall	0.17
Kidney	1.11	- content	0.09
Lung	1.35	Bone marrow–yellow	~LOQ
Spleen	0.24	- red	~LOQ
Heart	0.92	Spinal cord	1.13
Brain	0.95	Lymph node(s)	0.22
Muscle rump	0.58	Eye	0.29
- round steak	0.58	Ovary	0.14
- tenderloin	0.53	Adrenal gland	1.07
Fat perirenal	0.09	Thyroid gland	1.67
- subcutaneous	0.08	Pancreas	0.41
		Thymus	0.11

LOQ values: blood = 0.006 ppm; tissues 100 mg = 0.023 ppm; tissues 150-250 mg = 0.012 ppm; tissues 300-400 mg = 0.008 ppm.

Figure 13: Radioactivity in sheep blood after the oral administration of 10.5 mg [^{14}C]-triclabendazole/kg of bw.



The LOQ for the analytical method was 0.006 mg/kg triclabendazole equivalents**.**

Two Texel-cross sheep, one male and one female weighing 33 kg and 27 kg at dosing, respectively, were administered [^{14}C]-triclabendazole (specific radioactivity of 5.96 MBq/mg and radiochemical purity of 97.0%; - specific radioactivity of 6.13 MBq/mg and radiochemical purity of 98.5%) by oral gavage at a nominal dose rate of 10 mg/kg of bw (Ferguson, 1994b). Up to 168 hours after dosing, 77% and 4.7% of the administered dose was excreted in faeces and urine, respectively. Plasma protein binding determined *in vitro* in fortified samples and *ex vivo* in plasma collected from animals at 8 and 48 hours post-dosing was 99%. The animals were sacrificed 28 days after dosing, and the tissue distribution of radioactivity was determined. The concentration of radioactivity (in units of mg/kg equivalents of [^{14}C]-triclabendazole/kg) were 0.24 mg in forequarter muscle; 0.24 in tenderloin muscle; 0.24 in liver; 0.20 in kidney; 0.02 in subcutaneous fat; and 0.02 in renal fat.

Tissues from sheep orally dosed with [^{14}C]-triclabendazole and sacrificed at 28 days after dosing in the above study by Ferguson (1994b) were analysed in the context of a validation study for method REM 15/83 (Dieterle and Kissling, 1995). Extractability with dichloromethane was 91% (muscle) and 78% (liver), and the accountability (not corrected for procedural recoveries) of method REM 15/83 with UV detection was 32% (muscle) and 19% (liver) of the total residues for sheep.

[^{14}C]-Triclabendazole was administered by oral capsule at a dose of 10 mg/kg of bw to a goat and a sheep weighing 42.5 kg and 28.5 kg, respectively (Hamböck, 1982). The animals were sacrificed 10 days after dosing. The extractability of residues with various organic solvents and with 0.01M aqueous phosphate buffer solution was low for both animals. By comparison, the percentage of [^{14}C]-residues extracted with dichloromethane from tissues that had been solubilized using 2N aqueous NaOH and then acidified to pH<3 were 85% (liver), 89% (kidney) and 82% (muscle) for the goat and 78% (liver), 79% (kidney) and 89% (muscle) for the sheep. Oxidation using hydrogen peroxide transformed 40% and 42% of [^{14}C]-tissue residues to the common moiety keto-triclabendazole (CGA-110 754) in muscle of the goat and sheep, respectively.

The sheep study of Ferguson (1994b) was continued by determining the metabolic patterns in urine and faeces collected up to 168 hours after dosing (Thanei, 1995b). Urine and faeces accounted for 4.7% and 77% of the administered radioactivity, respectively, and contained five and eleven metabolic fractions, respectively. Unchanged triclabendazole was not detected in urine but accounted

for 16% of the dose in faeces. The major metabolic pathways of triclabendazole in sheep were oxidation to the sulphoxide and ultimately to the sulphone, and hydroxylation in position 4 of the dichloro-phenyl-ring. Therefore, the metabolic pathways of triclabendazole in sheep and cattle are essentially the same.

Triclabendazole was administered intraruminally at a dose rate of 10 mg/kg of bw to sheep surgically fitted with a bile duct cannula (Hennessy, et al., 1987). The profiles of triclabendazole metabolites in plasma and bile were determined. In plasma, only triclabendazole sulphoxide and triclabendazole sulphone were present and were bound to plasma albumin. In bile, the major triclabendazole metabolites were hydroxylated in the 4 position and excreted predominantly as sulphate esters with lesser proportions as glucuronide conjugates. Of the administered triclabendazole dose, 9.7% was excreted as free metabolites in bile, 35.8% was excreted as conjugated metabolites, and 6.5% was excreted in urine.

The absorption of triclabendazole and triclabendazole sulphoxide at different dose rates (5 and 10 mg/kg of bw); in different formulations (aqueous suspensions and aqueous solution); and administered via different routes (oral, intraruminal and i.v.) was studied in sheep (Strong, et al., 1982). Preliminary information only was reported and this study was not considered further.

Plasma levels of triclabendazole and triclabendazole sulphoxide in sheep were measured following the administration of triclabendazole on two occasions and in different formulations (Strong, et al., 1983). The authors reported that intra-animal variability in the pharmacokinetic behaviour of triclabendazole was small whereas inter-animal variability was large. In addition, the absorption of triclabendazole from an aqueous suspension and from a peanut oil formulation was reported to be similar. Administering triclabendazole on two occasions 8 weeks apart resulted in unchanged plasma levels of triclabendazole sulphoxide and triclabendazole sulphone in individual sheep. Partial stimulation of the oesophageal groove reflex was reported to occur in one of 15 sheep with no closure of the oesophageal groove occurring in the remaining 14 animals. It is concluded that when doses of triclabendazole are administered orally to ruminating sheep, they will generally enter the rumen.

A rapid and simple HPLC method for estimating triclabendazole and its metabolites in plasma was reported (Sanyal, 1994). The method was used to determine the pharmacokinetics of intraruminally administered triclabendazole in five sheep and five goats. The values of C_{max}, T_{max}, AUC and $t_{1/2}$ were similar for the two species.

Twenty-four sheep were assigned to three groups (each n=8)(Strong et al, 1988). Group 1 was dosed orally with 10 mg triclabendazole/kg of bw; Group 2 was dosed orally with 7.5 mg levamisole hydrochloride/kg of bw plus 10 mg triclabendazole/kg of bw; and Group 3 with 7.5 mg levamisole hydrochloride/kg of bw. The plasma kinetics of neither triclabendazole nor levamisole were affected by the other compound.

Six sheep of different breed and sex, aged 1 to 5 years and weighing 36-61 kg were assigned to 3 groups (each n=2) (Mohammed Ali, et al., 1986). Group 1 was drenched orally with 10 mg triclabendazole/kg of bw; Group 2 with 10 mg triclabendazole and 10 mg fenbendazole per kg of bw; and Group 3 with 10 mg fenbendazole/kg of bw. Each treatment was subsequently administered to the other two groups at 4-weekly intervals. The pharmacokinetics of triclabendazole were not altered when administered with fenbendazole.

Goats

A three year-old lactating goat weighing 42.5 kg bw was dosed orally with 10.1 mg [^{14}C]-triclabendazole (specific activity 1.129 MBq/mg and radiochemical purity 99%) labelled at the carbon atom in position 2 of the benzimidazole ring system (Hamböck and Strittmatter, 1981). Blood, milk, faeces and urine were collected until 10 days after dosing when the goat was sacrificed. Radioactivity in all samples including tissues collected at slaughter was determined by liquid scintillation counting.

In blood, the peak level of radioactivity of 13.7 mg/kg triclabendazole equivalents was observed at 36 hours. The maximum concentration of radioactivity in milk was 1.8 mg/kg triclabendazole equivalents in the 8-24 hour sample post-dosing. The overall recovery of radioactivity was 103.9% with excretion in urine and faeces accounting for 2% and 98% of the administered dose, respectively. Faeces but not urine contained triclabendazole and its sulphoxide and sulphone. The distribution of radioactivity at 10 days after dosing was highest in liver (1.0 mg/kg triclabendazole equivalents) and thyroid gland (1.3 mg/kg triclabendazole equivalents); lower levels were observed for fat and blood (each 0.08 mg/kg triclabendazole equivalents), red bone marrow (0.06 mg/kg triclabendazole equivalents) and yellow bone marrow (<0.02 mg/kg triclabendazole equivalents).

TISSUE RESIDUE DEPLETION STUDIES

Residue Depletion Studies with Unlabeled Drug

Cattle

A group of Hereford cattle comprising 12 males and 12 females aged 7-10 months and weighing 168-367 kg was treated orally at a dose of 18 mg triclabendazole/kg of bw and retreated 28 days later (Adams, 2004a). This treatment regimen corresponded to the minimum re-treatment interval in the directions for use on the product label. Six animals were sacrificed at each of 14, 28, 42 and 56 days following the second treatment. Samples of muscle (tenderloin), kidney, liver and renal fat were collected and analysed by HPLC for triclabendazole residues, measured as keto-triclabendazole. The limit of quantitation of the analytical method was 0.05 mg/kg. The results, corrected for recovery, are shown in Table 13.

Table 13: Residues of triclabendazole determined as keto-triclabendazole, following oral treatment of cattlewith *Fasinex 100* at 18 mg triclabendazole/kg of bw.

Sampling time (DALT[1])	Concentration of residues of triclabendazole measured as keto-triclabendazole (µg/kg)[2]			
	Muscle	Liver	Kidney	Renal fat
14	194, 221, 237, 248, 254, 271	797, 845, 862, 871, 1084, 1413	476, 487, 514, 586, 706, 1169	<50, <50, 72, 74, 78, 132
28	104, 118, 128, 155, 159, 175	263, 300, 339, 377, 424, 489	109, 118, 118, 129, 133, 165	<50, <50, <50, <50, <50, 62
42	103, 109, 124, 129, 132, 162	149, 183, 219, 262, 269, 288	49, 53, 62, 69, 75, 89	<50, <50, <50, <50, <50, <50
56	70, 85, 87, 90, 104, 111	48, 91, 96, 103, 131, 142	<50, <50, <50, <50, <50, <50	na, na, na, na, na, na

[1]. DALT = days after last treatment. [2]. Corrected for recovery. na = not analysed

Residue data corrected for recoveries from the cattle study by Adams (2004a) were analysed by linear regression (Strehlau, 2004a) in accordance with the EMEA/CVMP guideline (EMEA, 1996). One-sided, upper 95% tolerance limits with 95% confidence were calculated for muscle, liver and kidney. Model assumptions were checked using diagnostic tests. The linear regression assumptions regarding homogeneity of variances and homogeneity of normal distribution of errors were valid for muscle, liver and kidney; the assumption of linearity was valid for liver only.

Another GLP-compliant residue depletion study (Study No. AA031, 2001) involving a pour-on application to beef cattle was reviewed by the Committee. The animal phase of this study was conducted at Armidale, NSW, Australia. Beef cattle (n=25; Hereford/Hereford × Angus; 15 females and 10 male castrates; 126-192 kg bw) were treated with a single pour-on application of 0.75 mg abamectin/kg of bw and 45 mg triclabendazole/kg of bw. Groups of 5 animals were sacrificed on days

14, 21, 28, 35 and 42 after application, and samples of fat (back and perirenal), liver, kidney and muscle were collected. All tissue samples were stored frozen until analysed for residues.

A further 5 animals (3 females and 2 male castrates; 114 to 166 kg bw) were treated with a single pour-on application of 1.5 mg abamectin/kg of bw and 90 mg triclabendazole/kg of bw.. The cattle were held in covered pens overnight after their treatment. On the following day, the cattle were returned to open grazing paddocks with animals from the different treatment groups being held in separate paddocks. Animals were sacrificed at 35 days post-treatment, and samples of fat (back and perirenal), liver, kidney and muscle were collected for residues analysis. The concentrations of triclabendazole residues in tissue samples were determined using a validated method and analyses were completed within 14 months of sample collection. The results are shown in Table 14.

Table 14: Residues of triclabendazole following a single pour-on application of *Genesis Ultra Pour-on Roundworm, Liver Fluke & External Parasiticide for Cattle* to beef cattle at a dose rate of 45 mg triclabendazole/kg of bw.

Treatment Regimen	Sampling time (DALT[1])	Concentration of triclabendazole residues (mg/kg)[2,3]				
		Muscle	Liver	Kidney	Back fat	Perirenal fat
Single pour-on application of 0.75 mg abamectin/kg b.w. and 45 mg triclabendazole/kg b.w.	14	0.08, 0.34, 0.57, 0.59, 0.82	1.32, 2.76, 2.99, 3.59, 4.18	0.75, 1.36, 1.85, 2.08, 2.52	0.35, 2.08, 2.59, 2.92, 4.07	0.29, 1.12, 1.49, 1.52, 10.83
	21	0.13, 0.17, 0.21, 0.44, 0.53	0.75, 1.26, 1.63, 1.74, 1.88	0.40, 0.85, 1.01, 1.08, 1.17	0.29, 0.73, 0.82, 0.91, 0.93	0.16, 0.41, 0.42, 0.46, 0.76
	28	0.12, 0.15, 0.26, 0.28, 0.35	1.33, 1.57, 1.80, 1.85, 2.72	0.56, 0.91, 0.97, 1.00, 1.20	0.33, 0.43, 1.06, 1.06, 1.25	0.23, 0.35, 0.59, 0.76, 0.78
	35	0.09, 0.14, 0.14, 0.15, 0.26	0.39, 0.86, 0.95, 0.97, 2.29	0.15, 0.41, 0.45, 0.48, 0.89	0.15, 0.21, 0.33, 0.39, 0.63	0.09, 0.11, 0.20, 0.26, 0.62
	42	0.15, 0.22, 0.27, 0.27, 0.42	0.72, 0.74, 0.90, 0.93, 2.23	0.28, 0.28, 0.36, 0.42, 0.96	<0.03, 0.10, 0.18, 0.27, 1.37	0.04, 0.05, 0.11, 0.16, 0.57
Single pour-on application of 1.5 mg abamectin/kg bw and 90 mg triclabendazole/kg b.w.	35	0.16, 0.16, 0.20, 0.25, 0.30	1.40, 1.54, 1.63, 1.86, 1.91	0.62, 0.67, 0.82, 0.98, 1.22	0.30, 0.42, 0.45, 0.51, 1.31	0.20, 0.26, 0.26,0.33, 0.85

$LOQ_{triclabendazole}$ (all tissues) = 0.03 mg/kg; [1].DALT = days after last treatment; [2]. Residue results have not been corrected for method recoveries; [3].The residue results are expressed as triclabendazole equivalents, which can be converted to keto-triclabendazole equivalents by multiplying by a factor of 0.916.

Another GLP-compliant residue depletion study (Study No. ANT 1274, 2002) using the same pour-on product was applied to beef cattle. The animal phase of this study was conducted at Armidale/Dangersleigh, NSW, Australia. Twenty cattle (Hereford or Angus cross breed; 10 females and 10 male castrates; 208-290 kg bw) were treated with a single pour-on application of 0.75 mg abamectin/kg of bw and 45 mg triclabendazole/kg of bw. The cattle were treated and held in covered pens for 48 hours post-treatment. Thereafter, the cattle were returned to open grazing paddocks, and were observed on a weekly basis. Groups of 5 animals were sacrificed on days 49, 56, 63 and 70 after application, and samples of fat (back and perirenal), liver, kidney and muscle were collected. All tissue samples were stored frozen until analysed for residues.The concentration of triclabendazole

residues in tissue samples were determined using a validated HPLC method and analyses were completed within 6 months of sample collection. The results are shown in Table 15.

Table 15: Residues of triclabendazole following a single pour-on application of *Genesis Ultra Pour-on Roundworm, Liver Fluke & External Parasiticide for Cattle* to beef cattle at a dose rate of 45 mg triclabendazole/kg of bw.

Treatment Regimen	Sampling time (DALT[1])	Concentration of triclabendazole residues (mg/kg)[2,3]				
		Muscle	Liver	Kidney	Back fat	Perirenal fat
Single pour-on application of 0.75 mg abamectin/kg bw and 45 mg triclabendazole/kg bw	49	ND, 0.03, 0.04, 0.11, 0.15	0.51, 0.55, 0.99, 1.15, 1.27	0.26, 0.28, 0.57, 0.73, 0.82	ND, 0.10, 0.14, 0.2, 0.33	ND, 0.12, 0.16, 0.31, 0.68
	56	ND, 0.07, 0.08, 0.10, 0.12	0.45, 0.72, 0.73, 0.76, 1.01	0.26, 0.37, 0.40, 0.46, 0.49	0.09, 0.19, 0.24, 0.30, 0.36	0.04, 0.05, 0.11, 0.32, 0.39
	63	ND, ND, 0.01, 0.03, 0.05	0.31, 0.35, 0.39, 0.66, 0.84	ND, 0.18, 0.20, 0.36, 0.43	ND, ND, ND, 0.16, 0.19	ND, ND, ND, ND, 0.04
	70	ND, ND, ND, ND, 0.01	0.35, 0.37, 0.41, 0.45, 0.63	ND, 0.19, 0.20, 0.31, 0.39	ND, ND, ND, ND, 0.15	ND, ND, ND, ND, ND

[1]. DALT = days after last treatment; [2]. Residue results have been corrected for method recoveries; [3]. The residue results are expressed as triclabendazole equivalents, which can be converted to keto-triclabendazole equivalents by multiplying by a factor of 0.916.

A GLP-compliant residue trial (Study EL-55021, 2004) was conducted in beef cattle in New Zealand, using 21 females, weighing between 106-148 kg. One animal was included as an untreated negative control while 4 groups, each of 5 animals, were treated with a single pour-on application of 0.68 mg of abamectin/kg of bw and 38 mg of triclabendazole/kg of bw. Samples of fat (perirenal), muscle, liver and kidney were taken from groups of animals sacrificed at 77, 91, 105 and 119 days after treatment and analysed by HPLC for triclabendazole residues. The results are presented in Table 16.

Table 16: Residues of triclabendazole following a single pour-on application of *Genesis Ultra Pour-on Roundworm, Liver Fluke & External Parasiticide for Cattle* to beef cattle at a dose rate of 38 mg triclabendazole/kg of bw.

Treatment Regimen	Sampling time (DALT[1])	Concentration of triclabendazole residues measured as keto-triclabendazole (mg/kg)			
		Muscle	Liver	Kidney	Perirenal fat
Single pour-on application of 0.68 mg abamectin/kg b.w. and 38 mg triclabendazole/ kg b.w.	77	<LOQ, <LOQ, <LOQ, 0.10, 0.13	<LOQ, <LOQ, <LOQ, <LOQ, <LOQ	<LOQ, <LOQ, <LOQ, <LOQ, <LOQ	<LOQ, <LOQ, <LOQ, 0.10, 0.13
	91	<LOQ, <LOQ, <LOQ, <LOQ, <LOQ	<LOQ, <LOQ, <LOQ, <LOQ, <LOQ	<LOQ, <LOQ, <LOQ, <LOQ, <LOQ	<LOQ, <LOQ, <LOQ, <LOQ, <LOQ
	105	<LOQ, <LOQ, <LOQ, <LOQ, 0.10	<LOQ, <LOQ, <LOQ, <LOQ, <LOQ	<LOQ, <LOQ, <LOQ, <LOQ, <LOQ	<LOQ, <LOQ, <LOQ, <LOQ, <LOQ
	119	<LOQ, <LOQ, <LOQ, <LOQ, <LOQ	<LOQ, <LOQ, <LOQ, <LOQ, <LOQ	<LOQ, <LOQ, <LOQ, <LOQ, <LOQ	<LOQ, <LOQ, <LOQ, <LOQ, 0.10

LOD = 0.03 mg/kg; LOQ = 0.1 mg/kg; [1]DALT = days after last treatment

Sheep

Twenty-four sheep comprising 12 males and 12 females, 7 months old and weighing 29-42 kg bw, were treated orally at a dose of 10–13 mg triclabendazole/kg of bw and assigned to four groups (each 3 males and 3 females) One group was sacrificed at each of 14, 28, 42 and 56 days following treatment. Samples of muscle (tenderloin), kidney, liver and renal fat were collected and analysed by HPLC for triclabendazole residues, measured as keto-triclabendazole. The limit of quantitation of the analytical method was 0.05 mg/kg. Results, corrected for recovery, are shown in Table 17.

Table 17: Residues of triclabendazole determined as keto-triclabendazole, following oral treatment of sheep with *Fasinex 50* at 10–13 mg triclabendazole/kg of bw.

Sampling time (DALT[1])	Concentration of residues of triclabendazole measured as keto-triclabendazole (µg/kg)[2]			
	Muscle	Liver	Kidney	Renal fat
14	111, 143, 148, 152, 171, 200	327, 353, 428, 473, 487, 503	200, 219, 228, 258, 265, 279	<50, <50, <50, <50, <50, <50
28	70, 99, 101, 117, 140, 144	106, 128, 148, 181, 183, 201	68, 73, 93, 99, 118, 122	na, na, na, na, na, na
42	50, 51, 60, 64, 80, 83	<50, <50, <50, <50, <50, <50	<50, <50, <50, <50, <50, <50	na, na, na, na, na, na
56	<50, <50, <50, 51, 54, 57	na, na, na, na, na, na	na, na, na, na, na, na	na, na, na, na, na, na

[1]. DALT = days after last treatment; [2]. Corrected for recovery; na = not analysed

Residue data corrected for recovery for muscle, liver and kidney from the sheep study by Adams (2004a) were analysed in accordance with the EMEA/CVMP guideline (EMEA, 1996) by Strehlau (2004b). Depletion curves were estimated and one-sided, 95% tolerance limits with 95% confidence limits calculated after a single dose and extrapolated to a repeated administration 28 days after the first dose. Model assumptions were checked using diagnostic tests with the exception of linearity for liver and kidney residues. The latter could not be checked because there were only two time points. The model assumptions tested were shown to be satisfied. The residue depletion curves and corresponding tolerance limits predicted on the basis of repeated administration 28 days after the first dose were presented.

Bound residues and bioavailability

Tissues originating from cattle (Ferguson, 1994a) and sheep (Ferguson, 1994b) which were sacrificed 28 days after oral dosing with 12 mg (cattle) or 10 mg (sheep) [^{14}C]-triclabendazole/kg of bw were lyophilised, homogenised and mixed with powdered standard rat chow in a ratio of 80/20 w/w and fed to bile duct-cannulated rats (Hassler, 1995). In addition, diet mixtures containing cattle kidney, and sheep kidney and liver, were homogenised in water and orally administered by syringe during the first 8 hours of access to the fortified rat chow. This assured uptake of the diet mixture by the bile duct-cannulated rats. Urine, faeces and bile were collected from the rats until they were sacrificed 2 days after commencing the diet. Tissues including abdominal fat, kidney, liver and skeletal muscle were sampled at necropsy. The faeces of rats accounted for 85% (liver), 68% (kidney) and 91% (muscle) of the radioactivity present in the respective cattle tissue ingested, and for 88% (liver), 88% (kidney) and 88% (muscle) of the radioactivity present in the respective sheep tissue ingested. The bioavailability of the radioactivity from cattle and sheep tissues is shown in Table 18.

Table 18: Recovery of radioactivity (% of administered dose) following administration of cattle and sheep tissues containing [^{14}C]-triclabendazole derived residues to male bile duct-cannulated rats.

Tissue	Cattle			Sheep		
	Liver[1]	Kidney[2]	Muscle[1]	Liver[2]	Kidney[2]	Muscle[1]
Number of male rats	5	4	5	4	6	3
Urine 0–48 h	0.9	0.4	1.7	1.5	0.5	1.0
Bile 0–48 h	6.3	9.3	1.4	4.9	5.6	2.8
Residues						
Tissue & carcass	1.6	4.0	0.7	1.8	1.0	1.7
Bioavailability (%)	8.8	13.7	3.7	8.2	7.0	5.5

[1]. Tissue specimens were lyophilised, homogenised and mixed with powdered standard rat chow in a ratio of 80/20 w/w in a blender; [2]. The diet mixture was suspended in water.

The bioavailability of radioactive residues of [^{14}C]-triclabendazole-derived compounds was investigated in Sprague Dawley rats (Hardwick, 2004b) using tissues collected in an earlier cattle study (Needham, 2004b). Muscle, liver and kidney tissues were freeze-dried, powdered and prepared as a thick paste; additionally, a suspension of lyophilised muscle was prepared in water. Bile duct-cannulated rats were allocated to three groups. One group (n=6) was administered bovine muscle as an oral paste for 24 h followed by an additional gavage dose of 0.5 g lyophilised muscle suspended in water. A second group (n=6) was administered bovine liver as an oral paste for 24 hours. A third group (n=3) was administered bovine kidney as an oral paste for 24 h. Urine, faeces and bile were collected for 24 h on three occasions up to 72 h when the rats were sacrificed. Muscle, liver, kidney and the gastrointestinal tract plus contents were collected at necropsy. The mean recovery of radioactivity (expressed as mean ± sd) following dietary exposure was 91.4 ± 17.3% of the administered dose for muscle; 116.7 ± 6.8% for liver; and 90.6 ± 1.7% for kidney. The recovery of radioactivity in urine, faeces and bile is shown in Table 19.

Table 19: Recovery of radioactivity (% of administered dose) following administration of cattle tissues containing [^{14}C]-triclabendazole-derived residues fed to bile duct-cannulated rats.

	Muscle administration	Liver administration	Kidney administration
Number of rats	(n=6)	(n=6)	(n=3)
Sample			
Urine	0.78 ± 0.79	0.53 ± 0.45	<LOQ
Faeces	72.5 ± 19.3	93.6 ± 9.4	87.3 ± 1.3
Bile	17.2 ± 7.7	19.2 ± 5.0	3.3 ± 0.4

Data are rounded values of mean ± sd

In the same study, radioactivity in bile was detectable 48 h following the cessation of dietary exposure to bovine muscle, liver and kidney (Table 20). The percent of the administered dose of liver recovered as radioactivity in bile was highest in the 48-72h sample, suggesting that the duration of collection was inadequate and the bioavailability of incurred residues in bovine liver was under-estimated.

Table 20: Recovery of radioactivity (% of administered dose) in the bile of bile duct-cannulated rats following the administration of cattle tissues containing [^{14}C]-triclabendazole derived residues.

	Muscle administration	Liver administration	Kidney administration
Number of rats	(n=6)	(n=6)	(n=3)
Time (h)			
24	4.2 ± 1.8	5.6 ± 2.0	1.8 ± 0.8
48	11.7 ± 6. 5	4. 7 ± 5.4	0.7 ± 0.6
72	1.2 ± 0.6	8.9 ± 5.3	<LOQ
Total	17.2 ± 7.7	19.2 ± 5.0	2.5 ± 1.5

Data are rounded mean ± sd

ESTIMATION OF DAILY INTAKE

Calculation of the Estimated Daily Intake (EDI) of triclabendazole residues requires data on the median concentrations of marker residues and the ratios of marker to total residues, the quantities of the food commodities consumed (as defined by the standard food basket) and the bioavailability of residues. The latter discounts unreleased and undissolved residues, thereby providing a more realistic estimate of dietary intake. The median concentration of the marker residue in a specified tissue is derived from the predicted value of the regression line at the same time point used for establishing the MRL. In the case of triclabendazole, the choice of time points is limited because the ratio of marker to total residue concentrations in cattle tissues is known only at day 28. The corresponding information in sheep is even more limited.

Data from the following studies in cattle and sheep were used to prepare the summary of the available information on the ratio of marker to total residue concentrations (Table 21).

Two ruminating calves, one female (Aberdeen Angus) 63 kg bw and one male (Friesian/Limousin cross) 96 kg bw at the time of dosing, received a nominal dose of 12 mg [^{14}C]-triclabendazole/kg of bw by gavage (Ferguson, 1994a). Both animals were sacrificed 28 days after dosing. Radioactivity was determined in liver, kidney, muscle (tenderloin, hindquarter and forequarter), perirenal fat, subcutaneous fat, plasma, and red cells. The tissues obtained in this study were later analysed in the context of a method validation study (Dieterle and Kissling, 1995). Concentrations of the marker residue in liver, kidney and muscle were determined for the male animal only.

A male ruminating calf of 91 kg bw at the time of dose administration was studied. A dose of 12.55 mg [^{14}C]-triclabendazole/kg of bw was administered by oral capsule and the animal was sacrificed on day 28 after treatment. Radioactivity was determined in liver, kidney, muscle, fat, blood, red blood cells, and plasma. Concentration of the marker residue was determined in liver, kidney and muscle (Needham, 2004b).

The above evaluation demonstrates that the ratio of marker to total residue concentrations is only known for liver, kidney and muscle of two young male animals of a small subpopulation with regard to age and bw.

A study similar in design to the study in cattle (Ferguson, 1994a) mentioned above was conducted in sheep (Ferguson, 1994b). Two sheep of 27 kg (female) and 33 kg (male) pre-dose bw were given a nominal dose of 10 mg [^{14}C]-triclabendazole/kg of bw orally by gavage. Animals were sacrificed 28 days after dosing. Radioactivity was determined in liver, kidney, muscle (hindquarter, forequarter, and tenderloin) and perirenal and subcutaneous fat. The concentration of marker residue was determined in muscle and liver of the male sheep in the context of the method validation study conducted by Dieterle and Kissling (1995). There were significant inconsistencies in the use of the specific

radioactivities for the calculation of total residue and also a major discrepancy in the total residue concentration given for liver in the two studies. The final results shown below were obtained following independent re-calculations of the data taking into account error propagation:

Ratio	Mean	Standard error
Muscle	0.400	0.104
Liver	0.248	0.011

A report providing very few details provides some limited information on the ratio of marker to total residue concentrations in muscle of goat and sheep sacrificed at an earlier time point after treatment. A goat of 42.5 kg bw and a sheep of 28.5 kg bw received a single oral dose of 10.1 and 10.5 mg/kg bw, respectively. The animals were sacrificed ten days following dosing. The reported ratio of marker to total residue concentrations in muscle was 0.4 in the goat and 0.42 in the sheep (Hamböck, 1982).

Table 21: Summary of available information on the ratio of marker to total residue concentrations.

Species	Bw (kg)	Dose (mg/kg of bw)	Days after dose administration	Ratio of marker to total residue concentrations		
				Liver	Kidney	Muscle
Bovine	96	12	28	0.19	0.24	0.41
	91	12.55	28	0.24	0.27	0.32
Ovine	33	10.45	28	0.25		0.4
	28.5	10.5	10			0.42
Caprine	42.5	10.1	10			0.4

The median concentrations of the marker residue in cattle tissues were based on data collected on day 28, the only day when the ratio of marker to total residue concentrations is known. Accordingly, two residue depletion studies with unlabeled drug in cattle were considered. One study involved the oral treatment of twenty-four Hereford cattle which was repeated 28 days later (Adams, 2004b). The second study involved the application of a pour-on to beef cattle (Study No. AA031, 2001). The samples collected in this study were stored frozen for up to 14 months prior to analysis; however, no stability data were provided to support the validity of these storage conditions. An earlier study (Needham, 2004b) demonstrated that the mean concentration of triclabendazole after 6 months frozen storage declined to 72% of the initial concentration. The data from the pour-on study were therefore considered to be unsuitable for the purpose of deriving median residues, or for recommending MRLs.

Data from the residue depletion study in cattle dosed orally (Adams, 2004b) were evaluated using the procedure adopted by the 66th meeting of the Committee (WHO Technical Report Series, No. 939, 2006). Accordingly, the points on the curve describing the upper one-sided 95% confidence limit over the 95th percentile and the linear regression line at day 28 were derived for muscle, liver and kidney. The results are shown in Table 22 as “Tol28” and “Median28”, respectively. Also shown in Table 22 is “f”, the inverse of the marker to total residue concentration ratio, for muscle, liver and kidney. The corresponding values for fat are, of necessity, conservative estimates because observed values are not available. The “true” median of the marker residue concentrations in fat may be about 50 µg/kg (compared with the conservative value of 100 µg/kg used in Table 22). The extrapolation shows that fat probably contributes <<5 % to the total intake; the conservative estimate of the median residue concentration in fat would therefore appear to be acceptable.

Table 22: Estimates of intakes based on the residue concentrations found in tissues of cattle on day 28 after treatment.

Estimates of dietary intakes							
Cattle	"Tol28"	"Median28"	Marker (µg/ person*day)	Total/ marker f	Intake total (µg/person* day	Bioavailability	EDI (µg/person* day)
	µg/kg						
Muscle	246	161	48.2	3.1	149.4	0.13	19.4
Liver	827	423	42.3	5.4	228.5	0.13	29.7
Kidney	390	173	8.6	4.2	36.2	0.13	4.7
Fat		100	5.0	2.5	12.5	0.13	1.6
Sum					426.6		55.5

The results show that MRLs established on the basis of the tolerance limits of the marker residue concentrations found on day 28 after the last treatment would result in the EDI significantly exceeding the ADI (0-180 µg/person per day) when bioavailability is not taken into account. However, when bioavailability is factored in, which results in a more realistic estimate of consumer intake, the EDI of 55.5 µg per 60 kg person represents 30.8% of the ADI.

Similar considerations were applied to the evaluation of the residue data for sheep. The available database for sheep is even smaller than in cattle since measurable quantities of the marker residue were only found on days 14 and 28 in kidney and liver. In fat, all concentrations were < 50 µg/kg.

Table 23: Estimates of intakes on the basis of the residue concentrations found in tissues of sheep on day 28 after treatment.

Estimates of dietary intakes							
Sheep	"Tol28"	"Median28"	Intake marker (µg/person *day)	Total/ marker f	Intake total (µg/person *day)	Bioavailability	EDI (µg/person *day)
	µg/kg						
Muscle	174	103	31.0	2.50	77.6	0.13	10.1
Liver	288	154	15.4	4.00	61.6	0.13	8.0
Kidney	164	93	4.7	4.20	19.6	0.13	2.6
Fat		50	2.5	2.50	6.3	0.13	0.8
Sum			51.1		165.1		21.5

In Table 23, the median residue for fat is an hypothetical value, which is intentionally conservative and with kidney, the conversion factor for cattle kidney is used. The combined contribution of the total intake of fat and kidney is about 15%. In sheep, the EDI accounts for approximately 92% of the ADI when the bioavailability of the residues is not considered, and less than 12% of the ADI when the bioavailability of residues is taken into account. The tolerance limits of the marker residue concentrations found on day 28 after treatment are therefore an acceptable starting point for the recommendation of MRLs.

METHODS OF ANALYSIS

A report by Adams (2004c) on the validation of an analytical method for the determination of triclabendazole residues in cattle and sheep tissues (liver, kidney, muscle, fat) was reviewed by the 66th meeting of the Committee. This was an up-dated version of a method considered by the 40th Committee. Tissues are initially digested with hot alkali solution to release bound residues, then acidified, cooled and extracted with dichloromethane. For fatty tissues, an additional step to remove lipids by hexane-acetonitrile partitioning is included. The extract is evaporated to dryness, then taken up in ethanol:glacial acetic acid (1:1) and heated following addition of hydrogen peroxide to oxidize

the residues to keto-triclabendazole (the marker residue, identified as 5-chloro-6-(2, 3-dichlorophenoxy)-benzimidozole-2-one in the report of the 40th Committee). After a further partitioning step and evaporation to remove acetic acid, the residues are dissolved in dichloromethane and loaded on an anion exchange solid phase extraction cartridge and eluted with isopropyl alcohol/dichloromethane (12% v/v). The dried eluate is dissolved in acetonitrile and injected into the liquid chromatograph, with separation on a reversed phase (C-18) column and UV-detection at 296 nm. Quantitation is by external standard curve. Performance characteristics determined for the method are summarised in Table 24. The limits of detection and quantification for the method are based on estimates from calibration curves. The lowest concentration to meet acceptable performance criteria was 0.05 mg triclabendazole equivalents/kg (corresponding to 0.046 mg keto-triclabendazole/kg).

Table 24: Summary of validation study results for analysis of triclabendazole residues by liquid chromatography in various edible tissue.

Species	Edible Tissue	Limit of Detection[1] (mg/kg)	Limit of Quantification[2] (mg/kg)	Mean Recovery (%)	Repeatability[3] (%)
Cattle	Muscle	0.012	0.036	81-100	2.1-8.5
	Liver	0.024	0.074	84-87	1.7-9.6
	Kidney	0.020	0.058	89-97	3.1-9.4
	Fat	0.007	0.020	78-90	5.8-12
Sheep	Muscle	0.014	0.041	80-102	3.9-5.8
	Liver	0.008	0.024	90-102	2.4-4.8
	Kidney	0.012	0.034	89-93	5.6-7.0
	Fat	0.015	0.042	79-102	1.0-7.4

[1]. Based on mean response of blank, plus 3 standard deviations; [2]. Based on mean response of blank, plus 10 standard deviations; [3]. Within run, measured at 0.050, 0.100 and 0.200 mg/kg

No endogenous substances present in extracts produced a response in excess of the limit of quantification for keto-triclabendazole in any tissue. Other benzimidazole drugs, such as fenbendazole, thiabendazole and albendazole were not detected.

It was noted that the detection wavelength of 296 nm limits potential interferences. Triclabendazole sulphoxide and triclabendazole sulphone were detected, as was the parent drug triclabendazole; however, all three compounds were fully separated by the chromatography conditions used in the method. These compounds would normally be oxidized to keto-triclabendazole during the analysis. A confirmatory method was proposed which uses a phenyl liquid chromatography column as an alternative liquid chromatography system. Limits of quantification were higher than for the original method and the information obtained does not provide sufficient evidence for structural confirmation.

The stability of residues of triclabendazole in cattle tissues, measured as keto-triclabendazole, was determined (Adams, 2004d) using incurred residues in the tissues from two animals collected in Study Y03/49 (Adams, 2004b). Three replicates of each tissue were analysed prior to storage and then at 1.5, 3 and 6.5 months after storage in a freezer room that was maintained at a temperature ranging from a maximum average of –8°C to a minimum average of –22°C over the time period of the study. The average results, corrected for recovery, shown in Table 25, demonstrate that the residues remain essentially stable during this time period, with some decrease (maximum 33%) being seen at the final time point.

Table 25: Stability of incurred triclabendazole residues in cattle tissues under typical frozen storage conditions.

	Residues measured as keto-triclabendazole, for analytical recovery (mg/kg)			
	0 months (pre-storage)	1.5 months	3 months	6.5 months
Muscle 1	0.23 ± 0.01	0.24 ± 0.00	0.21 ± 0.00	0.19 ± 0.02
Muscle 2	0.25 ± 0.01	0.24 ± 0.02	0.20 ± 0.01	0.17 ± 0.02
Kidney 1	0.48 ± 0.03	0.42 ± 0.01	0.43 ± 0.05	0.36 ± 0.02
Kidney 2	0.47 ± 0.03	0.47 ± 0.06	0.44 ± 0.04	0.41 ± 0.02
Liver 1	0.85 ± 0.04	0.80 ± 0.02	0.78 ± 0.15	0.70 ± 0.04
Liver 2	0.75 ± 0.04	0.76 ± 0.04	0.81 ± 0.05	0.62 ± 0.01

A study reported the steps in Method REM 3/38 for determining residues that are hydrolysable and oxidisable to keto-triclabendazole (CGA-110 754) (Giannone, 1983). The sample is hydrolysed under alkaline conditions at 99-100^{o}C and the entire hydrolysate extracted with dichloromethane under acidic conditions. The dichloromethane is evaporated to dryness and the residue dissolved in a mixture of acetic acid/ethanol and oxidised overnight with hydrogen peroxide at 90^{o}C. The mixture is acidified and keto-triclabendazole is partitioned into dichloromethane. Further cleanup of the residue is carried out on a Silica Gel column followed by a C_{18} Sep-Pak column prior to the final determination by HPLC on a LiChrospher Si 100 column. The limit of quantification of the method is 0.027mg/kg keto-triclabendazole, which corresponds to 0.03 mg/kg triclabendazole.

Residues hydrolysable and oxidisable to keto-triclabendazole were quantified by method REM 3/38 and compared with total radioactivity in muscle from a goat (Adams, 2004e). A goat weighing 42.5 kg bw was dosed orally with [^{14}C]-labelled triclabendazole at a rate of 10.12 mg/kg of bw and sacrificed at 10 days after dosing. Tissue samples were collected and stored at –20^{o}C. Incurred residues in muscle were investigated. Total radioactivity was measured by scintillation counting after combustion; keto-triclabendazole was determined by HPLC according to method REM 3/38 except the residue was not cleaned up using a Sep-Pak column. Residues determined by method REM 3/38 accounted for 32-39% of the total radioactivity present with goat muscle (Table 26).

Table 26: Comparison of total radioactivity and total residues determined by HPLC in muscle of a goat dosed with [^{14}C]-labelled triclabendazole and sacrificed 10 days later.

Sample	Total radioactivity calculated as triclabendazole equivalent mg/kg	Total residues[1] determined by HPLC with method REM 3/83	
		mg/kg	% of total radioactivity
1	0.44	0.17	39
2		0.15	34
3		0.14	32
4		0.14	32

[1]. Residues determined as keto-triclabendazole and converted to triclabendazole with the conversion factor 1.09.

A study of Method REM 15/83, which is a replacement for method REM 3/83, was reported (Giannone and Formica, 1983). The two methods are identical through all steps up to and including the cleanup of residues on a C_{18} Sep-Pak column. Following C_{18} Sep-Pak column cleanup, final determination of keto-triclabendazole with method REM 3/38 is carried out on a LiChrospher 100 column. With the replacement method REM 15/38, keto-triclabendazole is determined by HPLC on a LiChrospher Si 100 column (as for method REM 3/83) or by a column switching technique involving two LiChrospher Si 100 columns. Recovery data for the determination of keto-triclabendazole with

and without column switching are comparable (Table 27). The limit of quantitation for method REM 15/83 is 0.027 mg keto-triclabendazole or 0.03 mg triclabendazole per kg.

Table 27: Recovery of keto-triclabendazole in muscle, liver, kidney and fat of sheep and cattle

Tissue	Fortification (mg/kg)	Recoveries after Sep-Pak cleanup (%)		Recovery after column switching (%)
		Cattle	Sheep	Sheep
Muscle	0.1	109	85, 69, 95	97, 95
	0.5	76	87, 67, 70, 74	72, 79
Liver	0.1	71	82, 76, 68, 68	67, 75
	0.5	77	85, 70, 60, 66	76, 79
Kidney	0.1	80	80, 73, 83, 74	89, 98
	0.5	70	77, 72, 75, 67, 75	75, 76
Fat	0.1	69	53	71, 73, 68
	0.5	55	69	54, 60, 61

Further validation of the method for analysis of sheep and cattle tissues was provided in Study V05/24 (Adams, 2005). The study demonstrated no background interferences; confirmed that precision was ≤15% at concentrations >0.10 mg/kg; and demonstrated the stability of the residues under freeze/thaw conditions.

In Study Y04/51, the method was extended to the analysis of tissues from goats (Adams, 2004e). Results, shown in Table 28, are based on analysis of three replicates at each of three concentrations for the three tissues tested (muscle, liver, kidney).

Table 28: Recovery and precision for determination of keto-triclabendazole residues in goat tissues.

Tissue	Concentration of keto-triclabendazole (µg/kg)					
	50		100		100[1]	
	Recovery (%)	CV (%)	Recovery (%)	CV (%)	Recovery (%)	CV (%)
Muscle	99	2.2	102	2.2	95	4.1
Liver	110	13	97	3.4	91	11
Kidney	98	1.7	85	2.7	85	5.4

[1]. Fortified samples analysed after storage at room temperature for 16-24 hours.

The stability of residues of triclabendazole in sheep tissues, measured as keto-triclabendazole, was determined (Adams, 2004f) using incurred residues in tissues obtained from two animals in Study Y04/22 (Adams, 2004a). Three replicates of each tissue were analysed prior to storage and after 2 and 4 months of frozen storage. The storage temperature varied from –5°C to 21°C during the study. There was minimal change in the residue concentration during the period of storage (Table 29).

Table 29: Stability of incurred triclabendazole residues in sheep tissues under typical conditions of frozen storage.

Tissue	Residues measured as keto-triclabendazole corrected for analytical recovery (mg/kg)		
	0 months (pre-storage)	2 months	4 months
Muscle 1	0.17 ± 0.01	0.15 ± 0.00	0.16 ± 0.01
Muscle 2	0.13 ± 0.01	0.12 ± 0.00	0.11 ± 0.01

Kidney 1	0.25 ± 0.01	0.23 ± 0.01	0.24 ± 0.00
Kidney 2	0.17 ± 0.01	0.15 ± 0.02	0.15 ± 0.00
Liver 1	0.47 ± 0.02	0.41 ± 0.04	0.41 ± 0.05
Liver 2	0.34 ± 0.01	0.27 ± 0.02	0.28 ± 0.02

Two new methods (Study No. AA031, 2001; Study No. ANT1274, 2002) for determining triclabendazole residues in animal tissues (referred to below as method 1 and method 2, respectively) were submitted for review by the 70th Committee. These methods are similar to the method reported by Adams (2004c) discussed above. Briefly, both methods involved alkaline hydrolysis of tissue homogenates at 90-100^{o}C, followed by extraction with dichloromethane under acidic conditions. In method 1, the solvent extracts were cleaned up using liquid/liquid partitioning. No clean up step was included in method 2. In both methods, the extracts were then oxidised overnight with hydrogen peroxide at 85-90^{o}C. Subsequently, the keto-triclabendazole analyte was partitioned into dichloromethane before clean up on an SPE column (method 2 only), and quantitation by HPLC with UV detection at 295-297 nm. Residue levels (expressed in keto-triclabendazole equivalents) were determined using an external standard calibration curve. Validation data for the analytical methods were provided to demonstrate the linearity of detector response, recoveries from fortified samples, method precision, and the limits of quantitation and detection.The following validation parameters were investigated in Study No. AA031 (2001) for method 1: linearity, precision, accuracy, specificity, limit of quantitation and limit of detection. The validation results are presented in Table 30.

Table 30: Summary of validation study results for analysis of triclabendazole residues by liquid chromatography (method 1) in bovine tissues.

Validation parameter	Details of test	Tissue matrix	Fortification level (mg/kg)	Linearity (r^2)	Accuracy (% recovery)	Precision (% RSD)
Linearity	Calibration standards extracted from tissues	Muscle	0.1-2.5	0.9995	--	--
		Kidney	0.05-2.5	0.9998	--	--
		Liver	0.05-2.5	0.9997	--	--
		Fat	0.05-2.5	0.9994	--	--
Recovery	% Recovery from fortified tissue samples (n=3)	Muscle	0.007-0.46	--	74-112	--
		Kidney	0.02-1.0	--	85-121	--
		Liver	1.2-1.6	--	94-100	--
		Fat	0.015-2.3	--	95-158	--
Precision	Replicate analyses of fortified samples	Muscle	0.007-0.46	--	--	10.9
		Kidney	0.02-1.0	--	--	10.3
		Liver	1.2-1.6	--	--	2.3
		Fat	0.015-2.3	--	--	24.0
Specificity	Determine whether there are method interferences associated with tissue components or related compounds			No known interferences detected and chromatographic runs showed specificity for all tissue types. No interference due to fenbendazole or oxfendazole.		
LOQ	Limit of quantitation (mg/kg)			LOQ (all tissues) = 0.045 mg/kg		
LOD	Limit of detection (mg/kg)			LOD (all tissues) = 0.03 mg/kg		

The validation parameters investigated in Study No. ANT1274 (2002) for method 2 were linearity, recovery from fortified samples, precision and limit of quantitation. The validation results are presented in Table 31.

Table 31: Summary of validation study results for analysis of triclabendazole residues by liquid chromatography (method 2) in bovine tissues.

Validation parameter	Details of test	Tissue matrix	Fortification level (µg/tube)	Linearity (r^2)	Accuracy (% Recovery)	Precision (% RSD)
Linearity	Calibration standards extracted from tissues	Muscle	--	0.9999	--	--
		Kidney	--	0.9870	--	--
		Liver	--	0.9983	--	--
		Fat	0.19-25	0.9992-0.9998	--	--
Recovery	% Recovery from fortified tissue samples (n=4-6)	Liver	0.39-25.0	--	78-120	--
		Fat	0.39-6.25	--	62-121	--
Precision	Replicate analyses of fortified samples	Liver	0.4 3.1 25.0	--	--	20.5 6.6 5.1
		Fat	0.4 1.6 6.3	--	--	20.4 10.0 15.2
LOQ	Limit of quantitation (mg/kg)			Muscle Kidney Liver Fat		0.13 1.25 0.39 0.19

It is noted that the LOQ for kidney of 1.25 mg/kg is anomalously high. The validation data for both methods are acceptable.

APPRAISAL

The 17th Session of the Codex Committee on Residues of Veterinary Drugs in Foods (CCRVDF) requested that the MRLs for triclabendazole in cattle and sheep be re-evaluated, including reconsideration of the data on bioavailability. No new studies on pharmacokinetics or metabolism were provided for evaluation; however, three new residue studies in cattle using a pour-on formulation were submitted. In its re-evaluation of the MRLs for triclabendazole in cattle and sheep, the Committee therefore re-evaluated the pharmacokinetic and metabolism data considered at the 40th and 66th meetings; evaluated the three new studies in cattle and re-evaluated the residue studies considered by the previous meetings; and reconsidered the studies which investigated the bioavailability of incurred residues of triclabendazole. This monograph reports the Committee's considerations and MRL recommendations for triclabendazole in cattle and sheep. The recommended MRLs were derived using the procedure adopted by the 66th meeting of the Committee (WHO Technical Series Report, No. 939, 2006).

Re-evaluation of the pharmacokinetic and metabolism data considered at the 40th and 66th meetings of the Committee confirmed the earlier findings. The ratio of marker residue concentration to total residue concentration in cattle tissues (muscle, liver and kidney) and sheep tissues (muscle and liver) on day 28 were derived from the metabolism studies and are shown in Table 21.

The modelling of dietary intake of residues present in cattle tissues was conducted at day 28, the only day when the ratio of the marker residue concentration to total residue concentration is known. The results of modelling show that the bioavailability of residues must be taken into account when establishing cattle MRLs; otherwise the EDI exceeds the ADI. Three studies (Hassler, 1995; Hardwick, 2004b; Needham, 2004a) on bioavailability were evaluated by the 66th meeting of the

Committee and were reconsidered by the present Committee. Only the study by Needham (2004) was suitable for determining the absolute bioavailabilities of [^{14}C]-triclabendazole administered by gavage, and of [^{14}C]-triclabendazole residues derived from lyophilised cattle tissues in the diet, and in turn, the bioavailability of [^{14}C]-triclabendazole-derived residues in lyophilised cattle tissues relative to the bioavailability of [^{14}C]-triclabendazole administered by gavage. Measurements of areas under the radioactivity-time curve indicated that the absolute bioavailability of [^{14}C]-triclabendazole approximated 70% when administered by oral gavage to rats. It is important to note that this value was used when establishing the ADI for triclabendazole. The absolute bioavailability of incurred residues in cattle tissues was the highest for liver at 9.2%. Based on these values, the bioavailability of incurred liver residues in cattle was calculated to be 13% (9.2/70 x 100) relative to gavage administration. Studies by Hassler (1995) and Hardwick (2004b) using the bile duct-cannulated rat model confirmed that the bioavailability of incurred residues from liver was higher than for muscle or kidney. However, the relative bioavailability could not be calculated based on the data from these studies. Therefore, the relative bioavailability for liver of 13% was used in the calculation of the EDI, as it represents the worse-case scenario.

Three new residue depletion studies involving pour-on applications to cattle were provided (Study No. AA031, 2001; Study No. ANT1274; Study EL-55021, 2004). In these studies, animals were sacrificed at days 14, 21, 28, 35 and 42; days 49, 56, 63 and 70; and days 77, 91, 105, and 119, respectively. For the purpose of recommending MRLs using the procedure adopted at the 66th meeting of the Committee, the ratio of marker to total residue concentrations must be known at the time point under consideration. In the case of triclabendazole, such information is available for day 28 only and in this regard, only the first of the three pour-on studies analysed tissues sampled on day 28 after the last treatment. In this study, however, samples were stored frozen for up to 14 months prior to being analysed for residues, and no data were provided from studies which investigated the stability of residues stored for this duration. Earlier studies by Needham (2004b) and Adams (2004d) found that triclabendazole residues in liver declined to 72% and 83% of the initial concentration, respectively, after 6 months of storage. On account of the uncertainty surrounding the stability of residues when stored frozen for 14 months, the data from Study No. AA031 were not considered suitable for the purpose of recommending MRLs.

A residue depletion study (Adams, 2004b) evaluated at the 66th meeting of the Committee was reconsidered. Bioavailability of incurred residues was taken into account and MRLs were recommended using the procedure adopted at the 66th meeting of the Committee (FAO/WHO, 2006). The sponsor's statistician noted: "The linear regression assumptions regarding homogeneity of variances and of normal distribution of errors are met for the muscle, liver and kidney. The assumption of linearity is solely met for liver." Nevertheless, this approach was used with minimal numerical differences (0.6-1.7%) in the calculated tolerance limits, compared to the present evaluation. The results of the study are summarised in Figure 14.

Figure 14: Depletion kinetics of residues convertible to keto-triclabendazole in tissues of cattle treated orally with a nominal dose of 18 mg triclabendazole/kg of bw on two occasions at an interval of 28 days.

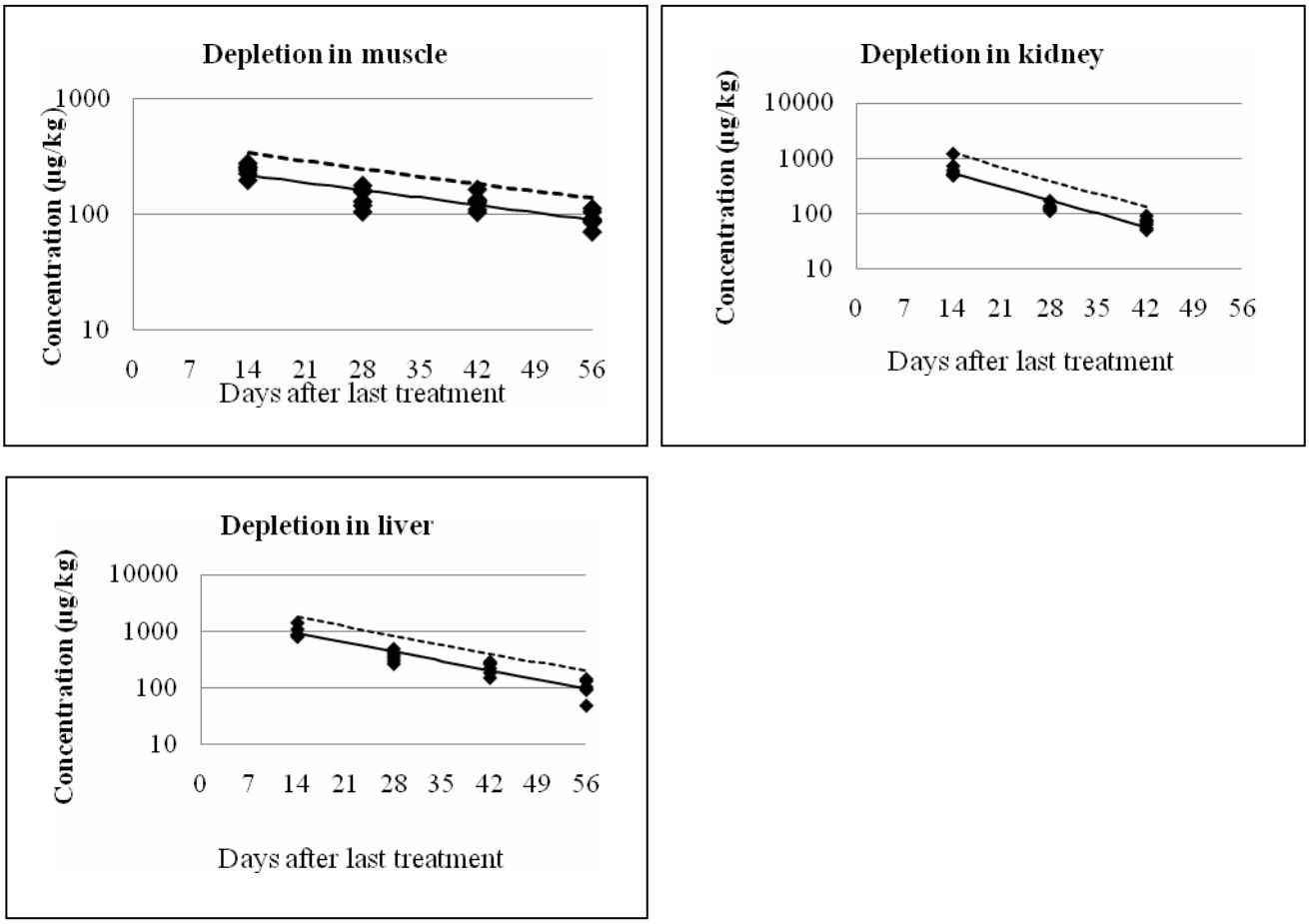
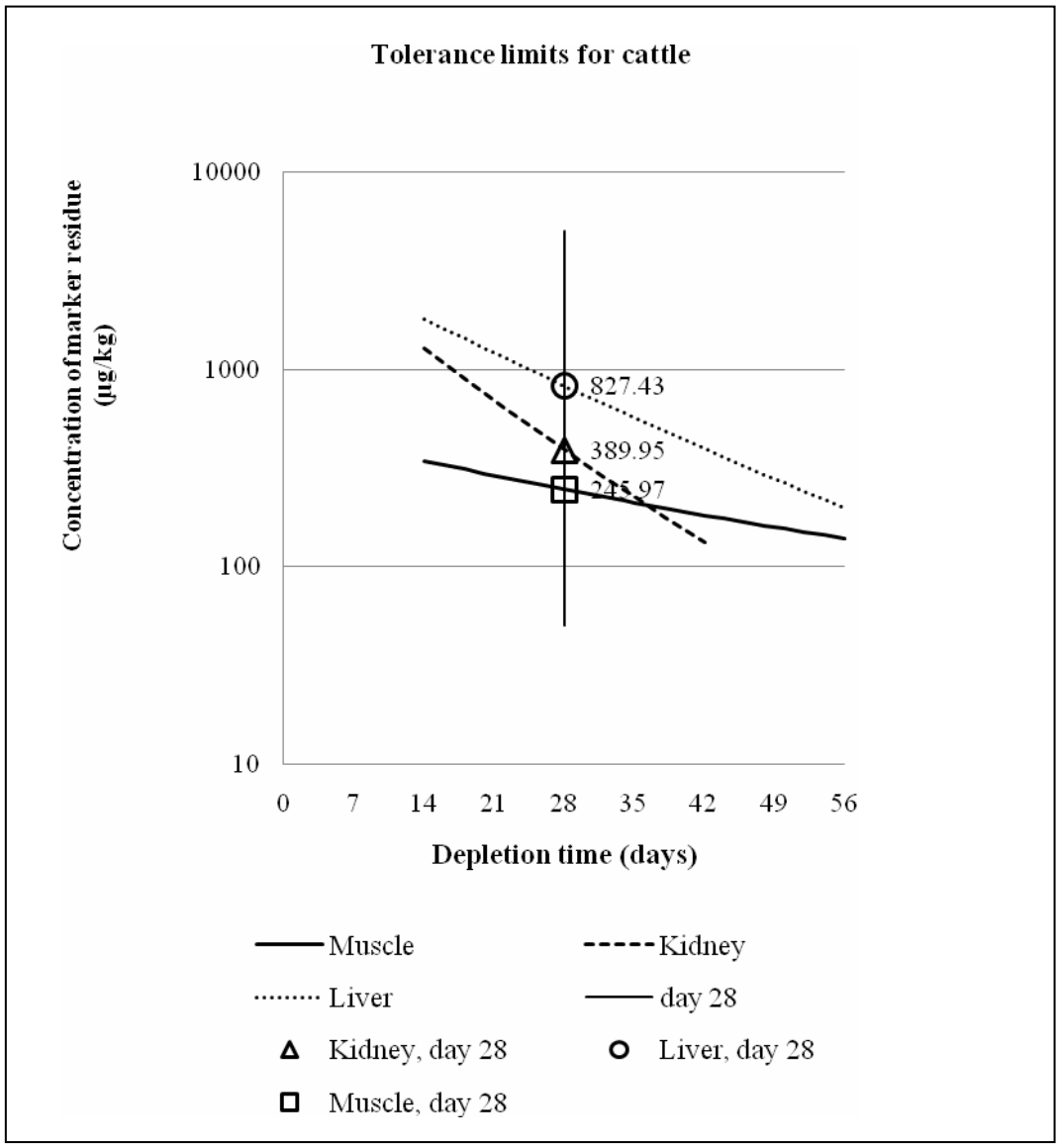


Figure 15 shows only the tolerance limit curves for the concentration of marker residue in liver, kidney and muscle of cattle. It also highlights the corresponding values for day 28, the only day when the ratios of the concentrations of marker and total residue are known. This is the only day for which dietary intake estimates are possible.

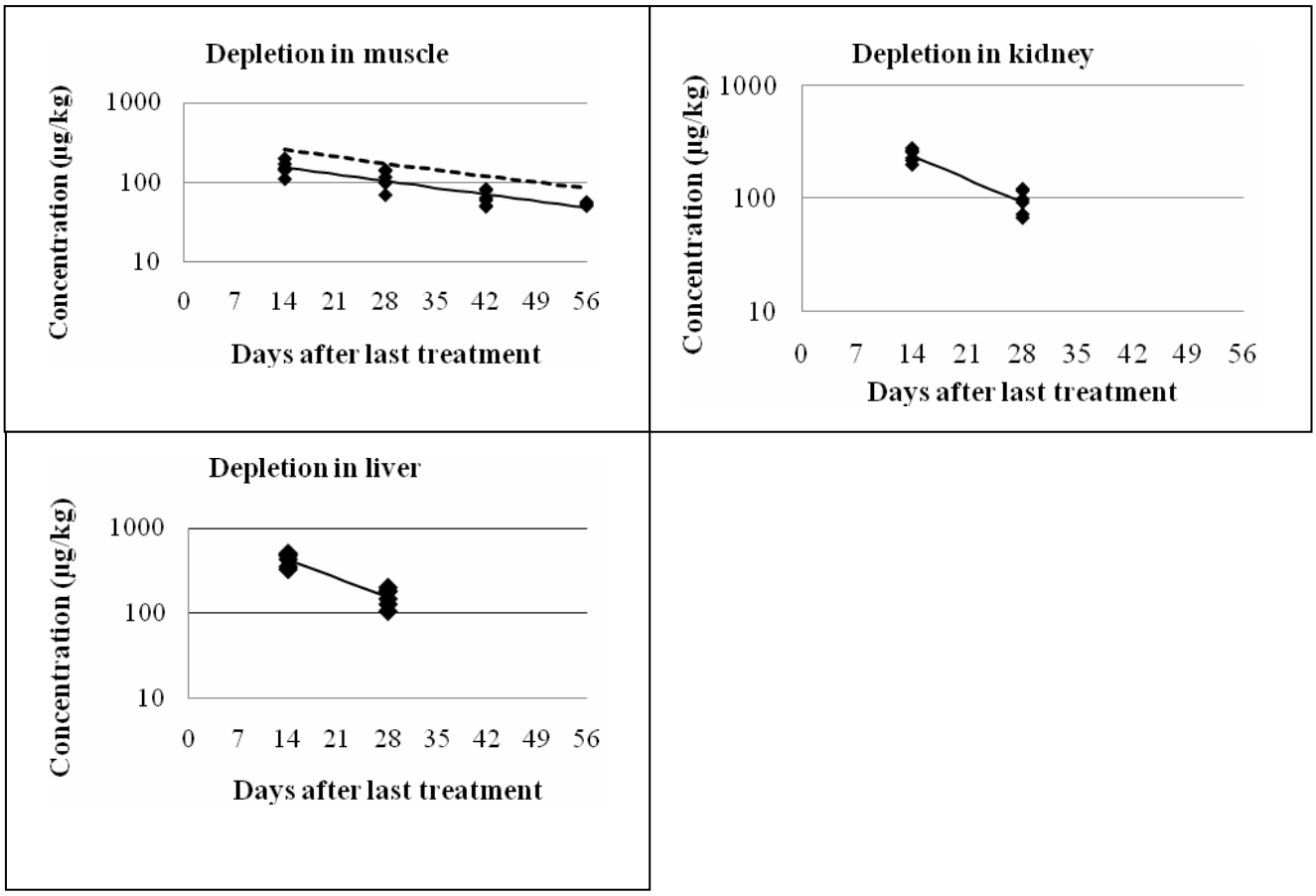
Figure 15: Tolerance limit curves for the concentration of marker residue in tissues of cattle and the concentration of marker residue in these tissues at day 28.



The proportions of the tolerance limits calculated for day 28 are 827:390:246 for liver:kidney:muscle. Table 22 shows some model calculations from the perspective of MRLs in cattle.

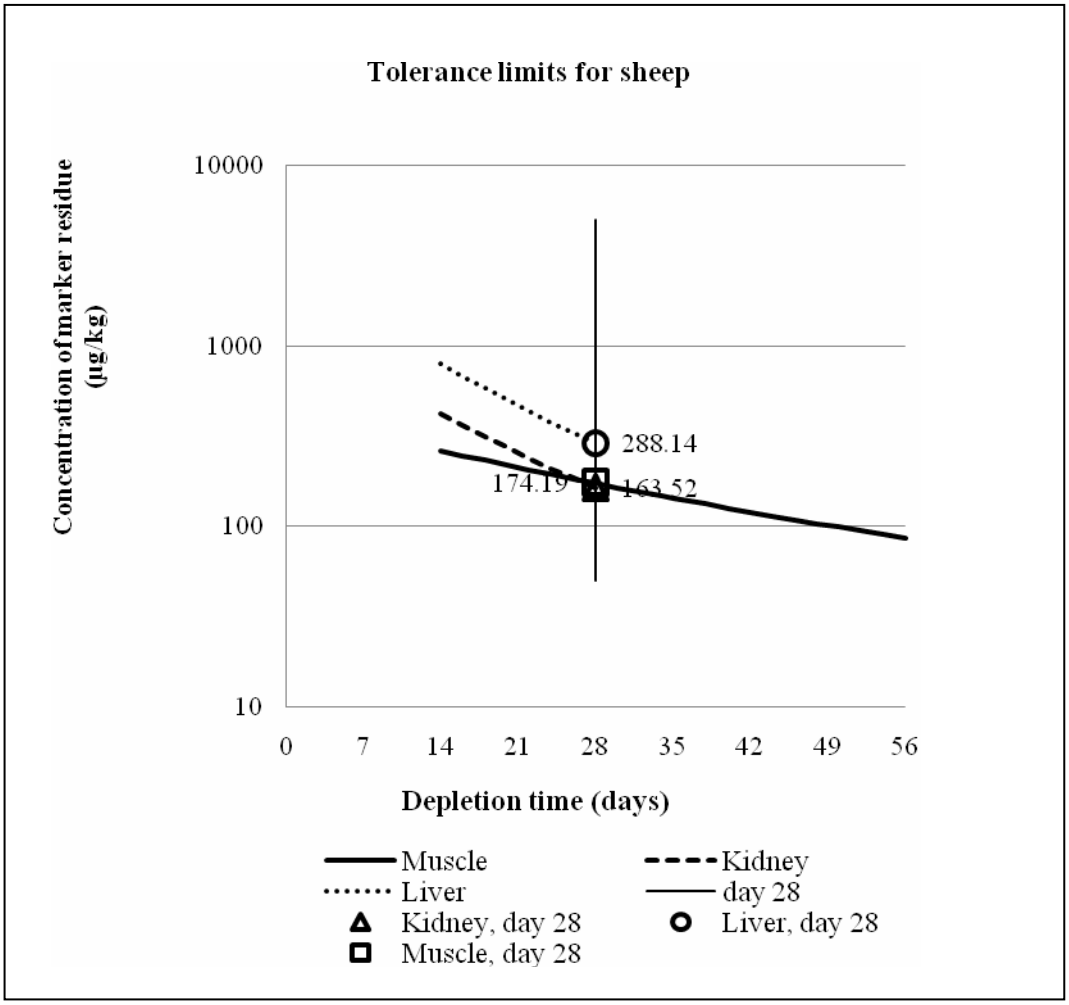
Similar considerations to those described above for cattle were applied to the evaluation of the sheep residue data. Figure 16 shows the depletion of marker residue in tissues of sheep.

Figure 16: Depletion kinetics of residues convertible to keto-triclabendazole in sheep tissues treated with a single oral dose of 10.5-13 mg triclabendazole/kg of bw.



The available database for sheep is smaller than that of cattle as measurable quantities of the marker residue were only found on days 14 and 28 in kidney and liver. In fat, all concentrations were < 50 µg/kg. Figure 17 summarises the results of statistical data analysis which is analogous to Figure 15.

Figure 17: Tolerance limit curves for the concentration of marker residue in tissues of sheep and the concentration of marker residue in these tissues on day 28.

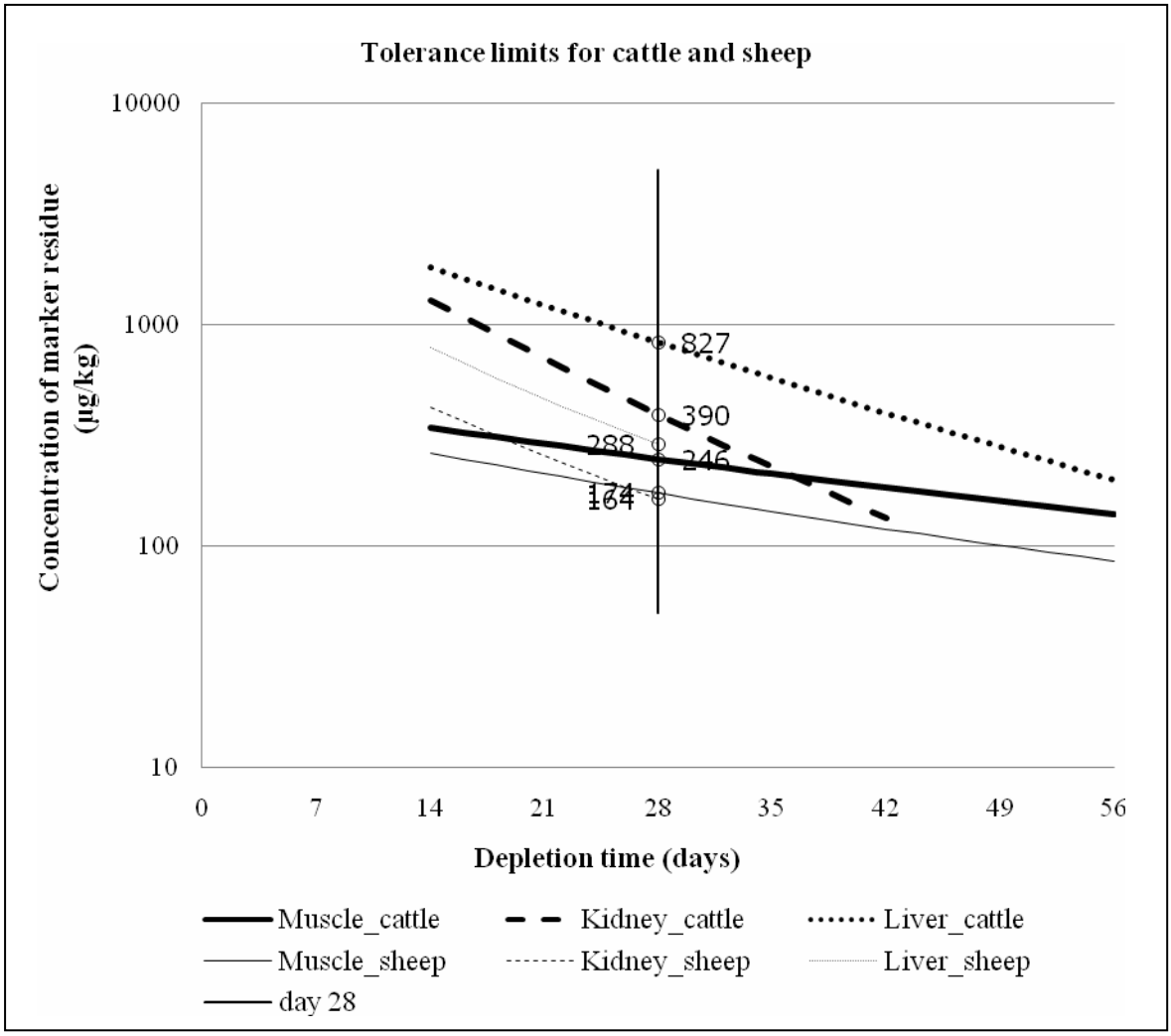


The proportions of the tolerance limits calculated for day 28 are 288:164:174 for liver:kidney:muscle. Table 23 shows calculations from the modelling of MRLs in sheep.

The dietary intake estimate shows with sheep, the tolerance limits of the marker residue concentrations found on day 28 after treatment are suitable for the establishment of MRLs.

Figure 18 combines the results of the depletion studies in cattle and sheep. The graph shows that the kinetic behaviour of triclabendazole is distinctly different in cattle and sheep and that there is no basis for establishing MRLs of identical numerical values for the two species.

Figure 18: Tolerance limit curves for the concentration of marker residue in tissues of cattle and sheep.



MAXIMUM RESIDUE LIMITS

In recommending the MRLs, the Committee took into account the following factors:

- An ADI of 0-3 µg/kg of bw was established by the fortieth meeting of the Committee, equivalent to 0-180 µg for a 60 kg-person.
- The marker residue is the sum of all residues extracted and converted to keto-triclabendazole.
- Liver and muscle are suitable target tissues.
- A validated analytical method is available for analysis of triclabendazole residues in edible tissues of cattle and sheep.
- The bioavailabilities of [^{14}C]-triclabendazole and [^{14}C]-triclabendazole-derived incurred residues administered to rats by oral gavage was 70% and 9.2%, respectively. Based on these data, the relative oral bioavailability of incurred residues was 13%.
- In cattle, the ratios of marker residue concentration to total residue concentration were 0.32 for muscle, 0.19 for liver, 0.24 for kidney and 0.4 for fat on day 28. In sheep, the ratios were 0.4 for muscle, 0.25 for liver, 0.24 for kidney and 0.4 for fat (a conservative value based on that for fat from cattle).

- The kinetic behaviour of triclabendazole is distinctly different in cattle and sheep and there is no basis for establishing MRLs with the same numerical values for the two species.
- MRLs for liver, kidney and muscle from cattle and sheep were derived from the curve describing the upper one-sided 95% confidence limit over the 95th percentile of the residues of the marker residue keto-triclabendazole on day 28 after the last treatment and are thus higher than those recommended by the 66th meeting of the Committee, which were based on the time point of 56 days.
- MRLs for fat were based on twice the LOQ of the analytical method.

On the basis of the above considerations, the Committee recommended the following MRLs for edible tissues of cattle, expressed as the marker residue, keto-triclabendazole: muscle, 250 μg/kg; liver, 850 μg/kg; kidney, 400 μg/kg; and fat, 100 μg/kg. These values were derived from the curve describing the upper one-sided 95% confidence limit over the 95th percentile of the residues on day 28 after the last treatment. The latter are depicted as "Tol28" in Table 22.

The Committee also recommended MRLs for triclabendazole for edible tissues of sheep, expressed as the marker residue, keto-triclabendazole, as follows: muscle, 200 μg/kg; liver, 300 μg/kg; kidney, 200 μg/kg; and fat, 100 μg/kg. These values were derived from the curve describing the upper one-sided 95% confidence limit over the 95th percentile of the residues on day 28 after the last treatment. The latter are depicted as "Tol28" in Table 23.

The Committee calculated the EDI using the median concentrations of marker residues in cattle tissues at day 28. The data for cattle (shown in Table 22) and not sheep (shown in Table 23) were chosen for the EDI calculation because the concentration of median residues in all tissues was higher for cattle than for sheep. Accordingly, the EDI represents 47.4 % of the ADI.

Tissue	Median residue	Standard Food Basket	Total residue concentration/Marker residue concentration	Bioavailability	EDI
Muscle	160.6 μg/kg	0.3 kg	3.1	0.13	19.4 μg
Liver	423.1 μg/kg	0.1 kg	5.4	0.13	29.7 μg
Kidney	172.5 μg/kg	0.05 kg	4.2	0.13	4.7 μg
Fat	100 μg/kg	0.05 kg	2.5	0.13	1.6 μg
EDI					55.4 μg

The MRLs previously recommended by the sixty-sixth meeting of the Committee for triclabendazole for cattle and sheep were withdrawn. As the Committee recommended significantly different MRLs for cattle and sheep and upon reviewing the limited data base for residues in goats, the Committee concluded that there was insufficient data to extend the recommended MRLs for goats. Therefore, the MRL for goats recommended at the sixty-sixth meeting of the Committee were withdrawn.

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TYLOSIN

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Addendum to the monograph prepared by the 38th Meeting of the Committee and published in FAO Food and Nutrition Paper 41/4

IDENTITY

International nonproprietary name: Tylosin (INN-English)

European Pharmacopoeia name: (4R,5S,6S,7R,9R,11E,13E,15R,16R)-15-[[(6-deoxy-2,3-di-O-methyl-β-D-allopyranosyl)oxy]methyl]-6-[[3,6-dideoxy-4-O-(2,6-dideoxy-3-C-methyl-α-L-ribo-hexopyranosyl)-3-(dimethylamino)-β-D-glucopyranosyl]oxy]-16-ethyl-4-hydroxy-5,9,13-trimethyl-7-(2-oxoethyl)oxacyclohexadeca-11,13-diene-2,10-dione

IUPAC name: 2-[12-[5-(4,5-dihydroxy-4,6-dimethyl-oxan-2-yl)oxy-4-dimethylamino-3-hydroxy-6-methyl-oxan-2-yl]oxy-2-ethyl-14-hydroxy-3-[(5-hydroxy-3,4-dimethoxy-6-methyl-oxan-2-yl)oxymethyl]-5,9,13-trimethyl-8,16-dioxo-1-oxacyclohexadeca-4,6-dien-11-yl]acetaldehyde

Other chemical names: 6S,1R,3R,9R,10R,14R)-9-[((5S,3R,4R,6R)-5-hydroxy-3,4-dimethoxy-6-methylperhydropyran-2-yloxy)methyl]-10-ethyl-14-hydroxy-3,7,15-trimethyl-11-oxa-4,12-dioxocyclohexadeca-5,7-dienyl}ethanal

Oxacyclohexadeca-11,13-diene-7-acetaldehyde,15-[[(6-deoxy-2,3-dimethyl-b-D-allopyranosyl)oxy]methyl]-6-[[3,6-dideoxy-4-O-(2,6-dideoxy-3-C-methy-a-L-ribo-hexopyranosyl)-3-(dimethylamino)-b-D-glucopyranosyl]oxy]-16-ethyl-4-hydroxy-5,9,13-trimethyl-2,10-dioxo-[4R-(4R*,5S*,6S*,7R*,9R*,11E,13E,15R*,16R*)]-

Synonyms: AI3-29799, EINECS 215-754-8, Fradizine, HSDB 7022, Tilosina (INN-Spanish), Tylan, Tylocine, Tylosin, Tylosine, Tylosine (INN-French), Tylosinum (INN-Latin), Vubityl 200

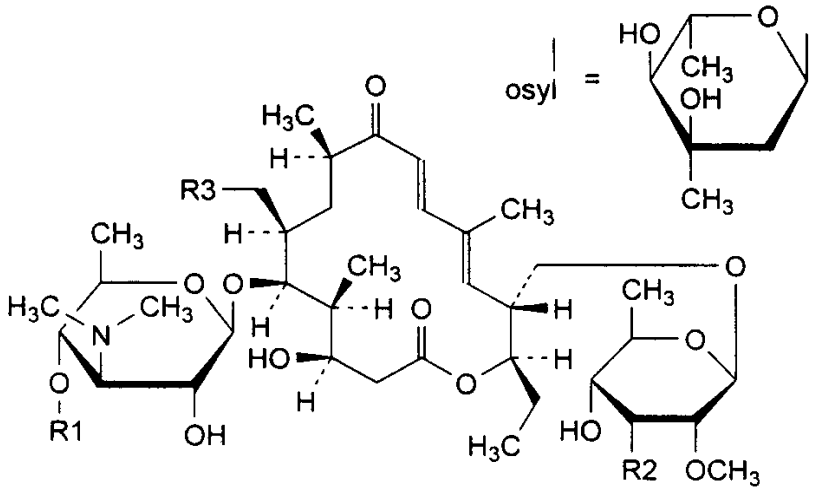
Chemical Abstracts System number: CAS 1401-69-0

Structural formula:

Tylosin is a macrolide antibiotic representing a mixture of four tylosin derivatives produced by a strain of Streptomyces fradiae (Figure 1). The main component of the mixture (> 80%) is tylosin A (M_r = 916; McGuire, et al., 1961). Tylosin B (desmycosin, M_r = 772; Hamill, et al., 1961), tylosin C (macrocin, M_r = 902; Hamill and Stark, 1964) and tylosin D (relomycin, M_r = 918; Whaley, et al., 1963) may also be present. All four components contribute to the potency of tylosin, which is not less than 900 IU/mg, calculated with reference to the dried substance (European Pharmacopoeia, 2004).

Relative antimicrobial activities of tylosin derivatives are: tylosin A – 1.0, tylosin B – 0.83, tylosin C – 0.75 and tylosin D – 0.35 (Teeter and Meyerhoff, 2003).

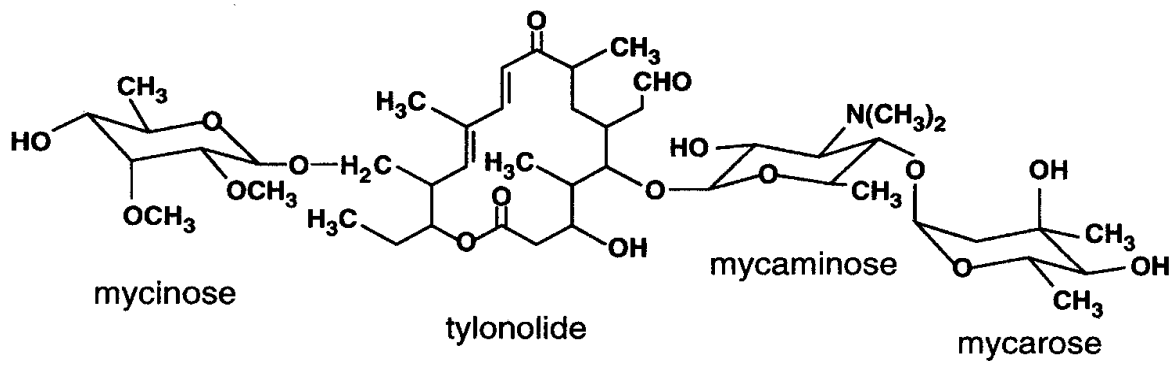
Figure 1: Chemical structure of tylosin.



Name	Mol. Formula	R1	R2	R3
tylosin A	$C_{46}H_{77}NO_{17}$	osyl	OCH_3	CHO
tylosin B	$C_{39}H_{65}NO_{14}$	H	OCH_3	CHO
tylosin C	$C_{45}H_{75}NO_{17}$	osyl	OH	CHO
tylosin D	$C_{46}H_{79}NO_{17}$	osyl	OCH_3	CH_2OH

Tylosin A contains a polyketide lactone (tylactone) substituted with three 6-deoxyhexose sugars (Figure 2). The addition of D-mycaminose to the aglycone is followed by concurrent ring oxidation at C-20 and C-23 (to generate the tylonolide moiety) and substitution with L-mycarose and 6-deoxy-D-allose. Bis-O-methylation of the latter generates mycinose and completes the biosynthesis of tylosin (Baltz, et al., 1983; Baltz and Seno, 1988).

Figure 2: Chemical structure of tylosin A.



Other pharmacologically active compounds, i.e., lactenocin, demecinosyl-tylosin (DMT) and O-mycaminosyl-tylonolide (OMT) have been isolated from fermentation media or aqueous commercial samples containing tylosin. In solutions for injections containing tylosin, an alkaline degradation product called tylosin aldol (TAD) has also been detected. Two epimers of this product, TAD1 and TAD2, as well as isotylosin A (isoTA) have been isolated (Paesen, et al., 1995abc). More recently,

two photoreaction products of tylosin in water, isotylosin A alcohol (isoTA1) and isotylosin A aldol (isoTA2) have been identified (Hu and Coats, 2007; Hu, et al., 2008).

Molecular formula: $C_{46}H_{77}NO_{17}$

Molecular weight: 916.1

OTHER INFORMATION ON IDENTITY AND PROPERTIES

Pure active ingredient: The main component of the mixture (> 80%) is tylosin A

Appearance: An almost white or slightly yellow crystalline powder

Melting point: 128-132°C

Solubility: 5 mg/ml (water 25°C), soluble in lower alcohols, esters, ketones, chlorinated hydrocarbons, benzene, ether, acetone, chloroform

UV Absorption: UV_{max} at 282 nm; Extinction coefficient ($E_{1\ cm}$ 1%) is 245 at 282 nm

Stability: Solutions are stable at pH 4-9 (maximum stability at pH 7);
Below pH 4 tylosin B (desmycosin) is formed as a result of acid hydrolysis, as occurs in honey;
In neutral and alkaline pH, tylosin aldol (TAD) is formed together with polar degradation products of unknown identity;
When tylosin solution is exposed to daylight, two photodegradation products, isotylosin A alcohol (isoTA1) and isotylosin A aldol (isoTA2), are formed

pKa: 7.73

log P (octanol-water): 1.63

RESIDUES IN FOOD AND THEIR EVALUATION

Tylosin was first evaluated by the Committee at the twelfth meeting (FAO/WHO, 1969). At that meeting, the Committee concluded that tylosin used in animal feed or in veterinary medicine should not give rise to detectable residues in edible products of animal origin. No ADI was established. Tylosin was subsequently evaluated at the thirty-eighth meeting of the Committee (FAO/WHO, 1991). Because of deficiencies in the toxicological and microbiological data, the Committee was not able to establish an ADI or recommend MRLs for tylosin. Before reviewing the compound again, the Committee requested the following information:

1. Detailed information from the reported reproduction and teratogenicity studies.
2. Studies designed to explain the positive result that was obtained in the mouse lymphoma genotoxicity assay in the absence of metabolic activation.
3. Studies designed to test the hypothesis that the increased incidence of pituitary adenomas in male rats after the administration of tylosin is a consequence of the greater rate of bodyweight gain in these rats.
4. Studies from which a NOEL for microbiological effects in humans can be determined.
5. Additional studies of residues in eggs using more sensitive analytical methods.
6. Additional information on microbiologically active metabolites of tylosin.
7. Studies on the contribution of the major metabolites of tylosin to the total residues in edible tissues of cattle and pigs.

In 2005, the 15th Session of the Codex Committee of Residues of Veterinary Drugs in Food (CCRVDF) requested that information on tylosin be submitted for evaluation by the sixty-sixth meeting of the Committee. However, none of the requested information was provided. In the absence of submitted information and in the light of a large number of scientific articles on tylosin appearing in the open literature since the thirty-eighth meeting of the Committee, a comprehensive review of the available information in the published literature concerning analytical methods, pharmacokinetics and tissue residues of tylosin in different animal species was carried out (Lewicki, 2006). However, the sixty-sixth meeting of the Committee did not critique the review as it considered published information alone was not suitable for conducting an evaluation of the compound. Tylosin was included on the agenda for the seventieth meeting of the Committee, as a result of a request from the 17th Session of the CCRVDF. Data as requested at the thirty-eighth meeting were provided for evaluation by the present meeting of the Committee.

Conditions of use

Tylosin is active against Gram-positive bacteria, mycoplasma and certain Gram-negative bacteria. Macrolide antibiotics are bacteriostatic compounds that reversibly bind to the 23S rRNA in the 50S ribosome subunit and inhibit mRNA-directed protein synthesis. They also stimulate the dissociation of peptidyl-tRNA from ribosomes during translocation. The precise mechanism of action has not been fully elucidated and many theories exist (Zhanel, et al., 2001; Gaynor and Mankin, 2005). It has been suggested that 16-membered-ring macrolides inhibit protein synthesis by blocking elongation of the peptide chain, but the 14- and 15-membered-ring macrolides are only potent inhibitors of mRNA-directed peptide synthesis (Retsema and Fu, 2001). It was also demonstrated that the 16-membered-ring macrolides (carbomycin, spiramycin and tylosin) inhibit peptidyl transferase, and the presence of mycarose was correlated with peptidyl transferase inhibition. However, tylosin B did not inhibit peptidyl transferase (Poulsen, et al., 2000). Results of comparative antibacterial evaluation of tylosin A and tylosin B showed that both compounds have almost identical antibacterial activity. In the same study, tetrahydro-desmycosin and dihydro-desmycosin showed decreased antimicrobial activity (Iveković, et al., 2003). Moreover, 4'-deoxy-10,11,12,13-tetrahydro-desmycosin, a derivative of tetrahydro-desmycosin, retained the antibacterial spectrum of tylosin with some improvement against tylosin-sensitive *Staphylococc*i and *Haemophilus influenzae* (Narandja, et al., 1995).

Tylosin is registered exclusively for veterinary use in several countries, primarily for use in the chronic respiratory disease (CRD) complex in chickens and infectious sinusitis in turkeys caused by *Mycoplasma gallisepticum*. Tylosin is also used to treat swine and bovine respiratory diseases and swine dysentery.

Dosage

Tylosin and its phosphate and tartrate salts are used in cattle, pigs and poultry for the treatment of infections caused by organisms sensitive to tylosin. Tylosin may be administered to calves orally in the drinking water, milk or milk replacer, at a daily dose of 10-40 mg/kg bw and to cattle by intramuscular injection at a dose of 5-20 mg/kg bw per day. In pigs, tylosin may be administered in the drinking water at a daily dose of 5-25 mg/kg bw; in the feed at a dose of 3-7 mg/kg bw per day; or by intramuscular injection at a dose of 5-20 mg/kg bw per day. In poultry, tylosin is used primarily in the treatment of chronic respiratory disease complex in chickens and infectious sinusitis in turkeys caused by *Mycoplasma gallisepticum*. It may be administered in the drinking water (0.5 g per litre) at a dose equivalent to 75 mg/kg bw per day; in addition, it may also be administered by intramuscular injection at a dose of 20-120 mg/kg bw per day (Plumb, 2002; Giguère, 2006). Tylosin is also approved for emergency use in the control of American foulbrood of honey bees at a dose of 200 mg/hive in 20g confectioners/powdered sugar once weekly for three weeks (FDA, 2005).

PHARMACOKINETICS AND METABOLISM

Tylosin is a highly lipid soluble, weak organic base (pKa = 7.73) that readily forms salts and esters. Available forms of tylosin are: tylosin base, tylosin tartrate and tylosin phosphate (McFarland, et al., 1997; European Pharmacopoeia, 2004). It is slightly to moderately bound to plasma proteins (30-47%) and is widely distributed in body fluids and tissues (Burrows, 1980). The volume of distribution (V_d) of tylosin is from 1-14.6 l/kg in various animal species. Although the comparative pharmacokinetics of tylosin in animals is poorly described in the scientific literature, reviews on tylosin pharmacokinetics are available (Wilson, 1984; WHO, 1991). Allometric relationships between tylosin total body clearance (Cl_B) and animal body weight have also been presented (Lewicki, 2006).

Absorption

Laboratory Animals

Rats

Tylosin is rapidly absorbed following oral administration to rodents. After a single oral dose of 50mg/kg bw of tylosin base or tylosin tartrate to rats, peak serum concentrations of tylosin of ≤1.0 µg/ml were seen after 1-2 hours. Within 7 hours, serum concentrations decreased to less than the limit of detection (LOD = 0.10 µg/ml) of the microbiological assay (WHO, 1991). Similar results were obtained in rats after intragastric administration of a solution of tylosin base. After a single dose of 20, 50 or 100 mg/kg bw of tylosin base, peak serum concentrations (about 0.5-1.1µg/ml) appeared after 2 hours (Kietzmann, 1985). When rats were given water mixed with a commercially available preparation of tylosin base (final concentration about 71 µg/ml), the bioassay of serum after 1-10 days of continuous medication revealed no detectable tylosin concentrations (< 0.1 µg/ml), while lung tissue contained 3.93-18.14 µg of tylosin/g (Carter, et al., 1987).

In rats, the reported V_d of 2.2 l/kg (Duthu, 1985) was similar to the values of V_d calculated for other animal species (for a review, see Lewicki, 2006). The elimination of tylosin from plasma is rapid in rats. Duthu (1985) reported a plasma elimination half-life ($t_{1/2}$) of 0.4 hour after intravenous administration and Cl_B of 86 ml/min/kg. A similar Cl_B of 70.9 ml/min/kg was observed for tylosin in mice (Cacciapuoti, et al., 1990).

Dogs

In dogs receiving tylosin orally by capsule at a dose of 1, 10 or 100 mg/kg bw/day for 8 days, tylosin blood concentrations determined 2 hours after the last dose ranged from < 0.15 µg/ml to 9. 5µg/ml (WHO, 1991). In another study, dogs receiving 25 or 100 mg/kg bw of tylosin base orally by capsule daily for 29 days demonstrated peak serum concentrations of 1.4-2.7 µg/ml at 2 hours after dosing at 25 mg/kg bw/day, and peak serum concentrations of 2.7-4.6 µg/ml at 2-5 hours after dosing at 100 mg/kg bw/day (WHO, 1991). In a separate study, there was no evidence of tylosin accumulation in the serum after 2 years of continuous administration of tylosin base in the diet (Anderson et al., 1966).

The magnitude and duration of tylosin blood levels following twice daily intramuscular injections with Tylocine Injection (tylosin 50mg/ml) was determined in Beagle dogs (van Duyn and Kline, Undated-a). Tylosin was administered at a dose rate of 11 mg/kg bw (5 mg/lb bw) 12-hourly for one day. Tylosin was rapidly absorbed after each injection producing two very similar blood level curves during the 24-hour period. Tylosin serum levels peaked at 1.9 and 1.7 µg/ml, respectively, at approximately 2 hours post-injection before declining to approximately 0.1 µg/ml at 10 hours.

A single intramuscular injection of 11 mg tylosin per kg bw was given to each of five dogs weighing 5-9 kg. Blood samples were collected at 0.5, 1, 2, 4, 6, 8, 10, 12, 24 and 48 hours after injection and the serum was assayed for microbiological activity. The mean concentrations of tylosin in blood were 2.5 µg/ml at 0.5 hour; 2.5 µg/ml at 1 hour; and 2.3 µg/ml at 2 hours after dosing. By 10 hours after

dosing, the tylosin blood level had declined to about. 0.1 µg/ml, the detection limit of the assay (van Duyn, et al., Undated).

In a study by van Duyn and Kline (Undated-b), six dogs were each given two intramuscular injections of 11 mg tylosin per kg bw 12 hours apart. Samples of blood were collected and assayed for tylosin activity at 2-hourly intervals up to 24 hours after the first injection. The peak blood concentrations of tylosin were 1.9 µg/ml and 1.7 µg/ml, occurring approximately 2 hours after each injection. Tylosin was not detected after about 12 hours.

Weisel and coworkers (1977) investigated the pharmacokinetics of tylosin in dogs after a single intravenous dose of tylosin. These authors reported values of 1.7 l/kg for V_d, 21.9 ml/min/kg for Cl_B, and 0.9 hour for plasma $t_{1/2el}$.

Food Producing Animals

Cattle

Peak blood concentrations of tylosin in cows were reached in 2-4 hours following intramuscular injection of tylosin base in 50% propylene glycol, or an aqueous solution of the tartrate salt (Sauter, et al., 1962; Gingerich, et al., 1977). In calves receiving tylosin base at a dose of 17.6mg/kg bw, peak concentrations of tylosin ranging from 2.07 to 2.3 µg/ml were observed 2 hours after intramuscular injection (van Duyn and Folkerts, 1979). Intratracheal administration of tylosin base at a dose of 25 mg/kg bw to calves resulted in peak serum concentrations of 5.2-5.8 µg/ml tylosin 1 hour after dosing. With intramuscular and subcutaneous injections, peak concentrations of 2.7-4.7 and 1.25-1.8 µg/ml, respectively, were reached 2 and 8 hours after injections (Hjerpe, 1979). In a separate study, peak serum concentrations of tylosin occurred about 5-6 hours after intramuscular injection of cattle, with systemic bioavailability of 70-80% of the administered dose (Ziv and Sulman, 1973; Baggot, 1978). The absorption of tylosin base following intramuscular injection was 17% and 94% complete after 7 hours and 24 hours, respectively (Nouws and Ziv, 1977a).

Kiorpes (1993) reported the relative bioavailability of Tylan® 200 Injection when administered subcutaneously and intramuscularly to cattle, using a cross-over study design. Two groups comprising six animals of each sex were administered 17.6 mg/kg bw Tylan® 200 Injection either subcutaneously or intramuscularly for five consecutive days. Serum samples were assayed for tylosin antimicrobial activity using a validated microbiological method; the limit of quantitation (LOQ) of the analytical method was approximately 0.1 mg/l. Following subcutaneous administration, tylosin was more slowly absorbed and attained a C_{max} of 0.89 mg/l, approximately one-half the C_{max} of 1.80 mg/l obtained after intramuscular administration. The time to reach maximum concentration (T_{max}) was 1 hour and 4.1 hours for intramuscular and subcutaneous administration, respectively, and the mean $t_{1/2}$ values were 6.9 hours and 16.2 hours, respectively. The bioavailability of tylosin following subcutaneous administration was 113% relative to intramuscular administration (Kiorpes, 1993).

The pharmacokinetics of tylosin in calves has also been reported by Abdul-Karim (2006a). Blood plasma concentrations after intravenous (10 mg/kg bw) and oral administration by gavage (20 mg/kg bw twice daily for five days) were determined by LC-MS/MS (the LOQ of the analytical method was 5 µg/kg) and the pharmacokinetic parameters were presented. Following the intravenous dose, C_0 was 16.9 µg/ml; V_{dss} was 3.49 l/kg; Cl_B was 23.0 ml/min/kg; $AUC_{0\text{-}24h}$ was 7.4 µg·h/ml; and $t_{1/2el.}$ was 10.9 hours. While recognising that estimates were based on limited data, oral bioavailability of tylosin in water is very low (about 1.7%). In the same study, the kinetics of tylosin plasma concentration were evaluated in cattle after a single intravenous injection (10 mg/kg bw) and intramuscular injections for three consecutive days (10 mg/kg bw per day). Pharmacokinetic parameters determined for the intravenous dose were: C_0 = 31.3 µg/ml; V_{dss} = 2.01 l/kg; Cl_B = 10.0 ml/min/kg; $AUC_{0\rightarrow\infty}$ = 16.8 µg·h/ml; and $t_{1/2el.}$ = 12.7 hours. Pharmacokinetic parameters determined for the final intramuscular dose were: C_{max} = 2.1 µg/ml; T_{max} = 2.6 hours; $t_{1/2el.}$ = 16.6 hours; and $AUC_{0\rightarrow\infty}$ = 18.4µg·h/ml. The

bioavailability of tylosin following intramuscular administration was 110% relative to intravenous administration (Abdul-Karim, 2006a).

The administration of tylosin base as a single intravenous or intramuscular injection to cattle at a dose of 4.6-7.3 mg/kg bw has been reported (Nouws and Ziv, 1977b; Nouws and Ziv, 1979). Tylosin concentrations in bile were 59.1 µg/ml (i.v.) and 56.3 µg/ml (i.m.) at 7 hours after injection; 35.1 µg/ml (i.m.) at 24 hours after injection; and 12.1 µg/ml (i.m.) at 31 hours after injection. The bile:serum concentration ratios were 296:1 (i.v.) and 62:1 (i.m.) at 7 hours after injection; 100:1 (i.m.) at 24 hours after injection; and 48:1 (i.m.) at 31 hours after injection. These ratios were much lower than the range of 1230-3780:1 reported for the dog (WHO, 1991). In the cattle study, tylosin concentrations in urine were 29.7 µg/ml (i.v.) and 41.7 µg/ml (i.m.) at 7 hours after injection; 12.9 µg/ml (i.m.) at 24 hours after injection; and 17.7 µg/ml (i.m.) at 31 hours after injection (Nouws and Ziv, 1977b; Nouws and Ziv, 1979).

In general, lipophilic weak bases such as tylosin readily pass from plasma to milk, which has a lower pH than plasma. This was confirmed in several experiments in different ruminant species. In cows receiving a single intravenous injection of tylosin tartrate at a dose of 20 mg/kg bw, peak concentrations of tylosin in milk (about 10 µg/ml) were observed 4 hours after injections; corresponding plasma concentrations of tylosin were about. 3.5 µg/ml. Lower peak values (about. 6 µg/ml) were observed in cows' milk 6 hours after a single intramuscular injection of tylosin tartrate at the same dose. When tylosin base was administered to cows intramuscularly at a dose of 12.5 mg/kg bw 12 hourly for 48 hours, the concentration of tylosin in milk peaked at about 7 µg/ml after 60 hours and then rapidly decreased to 1.5 µg/ml at 72 hours. Milk:serum concentration ratios corrected for differences in protein binding ranged up to about 20:1 (Gingerich, et al., 1977). Similar milk:serum concentration ratios up to 17.5:1 were observed in cows after a single intramammary infusion of 200 mg of tylosin/quarter. When mastitic cows received repeated intramuscular injections of tylosin base at a dose of 10 mg/kg bw every 12 hours for 5 days, concentrations of tylosin in milk steadily increased up to 18 µg/ml on day 5 after the onset of therapy (El-Sayed, et al., 1986).

Pigs

When pigs were administered tylosin tartrate orally at 30 mg/kg bw, tylosin activity was detected in plasma 10 minutes after dosing, with the maximum concentration of 2.4 µg/ml occurring approximately 1.5 hours later. A comparison of the blood AUCs following i.v. and p.o. administration provided an estimate of biological availability of 22.5%. When tylosin as the granulated phosphate was administered orally to pigs at a dose of 110 mg/kg bw, tylosin serum activity peaked 1 hour after dosing (average 17.8 µg/ml) and was not detectable (< 0.1 µg/ml) 24 hours after dosing. Similar results were obtained after the oral administration of tylosin phosphate in water at a dose rate of 50mg/kg bw Tylosin concentrations were detected in serum from 10 minutes to 8 hours after dosing and peaked 1 hour after dosing at 8.5 µg/ml (WHO, 1991). The results of a comparative residue study in pigs suggest that absorption of tylosin phosphate from the alimentary tract is comparable to that of tylosin tartrate (Iritani, et al., 1975).

Following intramuscular injection of pigs with tylosin base in 50% propylene glycol, or with an aqueous solution of the tartrate salt at a dose of 2.5-5 mg/kg bw, peak blood concentrations of tylosin were reached within 0.5-2 hours. Moreover, the results demonstrated that tylosin activity persisted in blood for up to 14 hours with the base, but only up to 8 hours with the tartrate salt (Sauter, et al., 1962). When pigs received tylosin base at a dose of 10 mg/kg bw by intramuscular injection, peak plasma concentrations of tylosin (0.4-1.9 µg/ml) were reached after 0.3-3 hours and bioavailability was 95% (Prats, et al., 2002a). Following a single intramuscular injection of pigs with a commercial mixture of tylosin and florfenicol (FTD-inj®) at doses of 2.5 or 10 mg/kg bw (tylosin) and 5 or 20 mg/kg bw (florfenicol), the C_{max} of tylosin was 1.3 µg/ml occurring at 2.4 hours for the low dose and 2.7 µg/ml occurring at 2.57 hours for the high dose. The $t_{1/2el}$ was 3.9 hours and 3.0 hours for the low and high doses, respectively (Kim, et al., 2008).

Tylosin levels in serum and lung tissue were measured in pigs following a single intramuscular dose of 17.6mg tylosin per kg bw as Tylan® 50. Tylosin was rapidly absorbed producing measurable serum and lung concentrations within 2 hours after the injection. Peak concentrations of 14.0 mg/kg and 2.0 µg/ml were observed in lung and serum, respectively, at 4 hours. Tylosin was not detected in serum after 12-24 hours but persisted in lung tissue for 48 hours (van Duyn and Johnson, Undated). In a similar experiment, tylosin was measured in serum and lung tissue of pigs following administration of a single intramuscular injection of Tylan® 50 at 8.8 mg/kg. Peak concentrations of tylosin occurred 2 hours after the injection and were 5.7mg/kg in lung tissue and 2.0 µg/ml in serum. Tylosin was detectable in serum for less than 12 hours and in lung tissue for 36-48 hours (van Duyn, Undated).

In another study comparing tylosin concentrations in lung and serum, pigs were injected intramuscularly with Tylan® 200 at a rate of 10 mg/kg bw for five consecutive days. The activity of tylosin residues was determined in sera and lung tissue by microbiological assay. Peak serum activity (1.7 µg/ml) occurred in pigs 2 hours after dosing and declined over 4 and 6-hours to 0.6 and 0.4 µg/ml, respectively. No activity was detected in sera sampled at 12 and 72 hours after dosing. Peak tylosin activity (5.8 mg/kg) in lung occurred 2 hours after injection. Tylosin activity in lung then declined, being below the LOQ of 0.1 mg/kg at 12 and 72 hours (Cochrane and Thomson, 1990).

Pratts and coworkers (2002a) reported the values of 4.5 hours for plasma $t_{1/2el.}$, 26.8 ml/min/kg for Cl_B and 14.6 l/kg for V_d for tylosin after single intravenous administration to healthy pigs. These authors also reported $t_{1/2el}$ of tylosin exceeding 24 hours when tylosin base was administered intramuscularly to pigs at a dose of 10 mg/kg bw.

Chicken/Poultry

When broiler chickens weighing 720 g received a single dose of 50 mg tylosin (as tylosin tartrate) per bird by stomach intubation, tylosin activity was detected in serum after 0.5 hour. Maximum serum concentrations of 0.6-4.0 µg/ml occurred after 2 hours, and serum concentrations were negligible after 24 hours. Following oral dosing of chickens weighing 2 kg at 1, 2, and 3 hours with 50 mg tylosin, maximum serum concentrations of about 0.3 µg/ml resulted at 4 hours after the last dose. Serum concentrations declined thereafter and were negligible at 24 hours after dosing (WHO, 1991). Similar results were obtained in chickens receiving a single oral dose of 10mg/kg bw of tylosin tartrate. A maximum plasma concentration of 1.2 µg/ml was observed 1.5 hours after tylosin administration and the oral bioavailability of tylosin was 30-34% in this study (Kowalski, et al., 2002). Ziv (1980) reported that chickens drinking water medicated with tylosin tartrate at rates of 500 and 700 mg/l for 48 hours had average serum concentrations of tylosin of 0.12 and 0.17 µg/ml, respectively. In this study, maximum concentrations of 0.2-0.3 µg/ml occurred after 24 hours (Ziv, 1980). In an oral bioequivalence study, two commercial products containing tylosin tartrate were compared on the basis of serum tylosin concentrations in 5- and 7-week old broilers and 9-month old layers. The birds were dosed with drinking water medicated with 750 mg tylosin tartrate/litre for 5 days. The rolling average tylosin concentration in serum approximated 0.20 µg/ml for each of the two commercial products (Ziv and Risenberg, 1991). In contrast to results obtained from pigs (Iritani, et al., 1975), tylosin phosphate was not as well absorbed as tylosin tartrate from the alimentary tract in chickens. No tylosin was detected in blood or muscle of chickens fed a diet containing tylosin phosphate up to 1500 mg/kg for eight weeks (Yoshida, et al., 1973).

In a more recent GLP-compliant study, the pharmacokinetics of tylosin in broiler chickens was investigated. Tylosin A was administered intravenously at 25 mg tylosin activity/kg bw as Tylan® Soluble; orally by gavage as an aqueous solution at 25 mg tylosin activity/kg bw as Tylan® Soluble; or orally by gavage in a feed slurry at 25 mg tylosin activity/kg bw as Tylan® Premix (Lacoste, 2003). The dose rates used in these studies conform to the recommended daily doses for chickens. Pharmacokinetic analysis of plasma concentration-time data for intravenous administration of Tylan® Soluble gave values for AUC and AUMC of approximately 7.1µg·h/ml and 35.8 µg·h^2/ml, respectively. The calculated mean residuence time (MRT) approximates 5.0 hours. The first phase $t_{1/2}$

calculated using α approximates 0.16 hour; the second phase $t_{1/2}$ calculated using β approximates 1.26 hours; and the terminal elimination phase $t_{1/2}$ calculated using γ approximates 35 hours. Pharmacokinetics analysis of plasma concentration-time data obtained after oral administration of tylosin yielded the following values: C_{max} = 0.4 µg/ml, T_{max} = 2 hours (Tylan® Soluble); and C_{max} = 0.2 µg/ml, T_{max} = 2 hours (Tylan® Premix). The absolute oral bioavailability calculated from the AUC_{total} and corrected from mean administered doses was approximately 11% for Tylan® Soluble and approximately 7% for Tylan® Premix (Lacoste, 2003).

A recent study assessed the kinetics of tylosin plasma concentrations in chickens after intravenously administering 10 mg/kg bw; orally administering 74 mg/kg bw per day in drinking water; and orally administering 92.5 mg/kg bw per day as a premix (Abdul-Karim, 2006b). The pharmacokinetic parameters were similar to those reported by Lacoste (2003). The bioavailability of tylosin in drinking water and as a feed premix was 3 and 8%, respectively. With intravenous doses, the initial mean plasma concentration (C_0) was 8.7 µg/ml, the mean Cl_B was 136ml/min/kg, and the mean V_{dss} was 8.6 l/kg (Abdul-Karim, 2006b).

Distribution

As mentioned above, tylosin is widely distributed in body fluids and tissues. Tissue:plasma concentration ratios of tylosin are reported to be 2.05:1 in cows and 2.5:1 in goats (Baggot and Gingerich, 1976; Atef, et al., 1991). In cows, the reported V_d of 1.1-2.27 l/kg (Ziv and Sulman, 1973; Baggot and Gingerich, 1976; Gingerich, et al., 1977; Cester, et al., 1993) is similar to that for sheep and goats. However, higher values of V_d for tylosin of 2.48-5.68 l/kg were reported for young calves (Burrows, et al., 1983; Burrows, et al., 1986).

Cattle

Tylosin base was administered intramuscularly to cows at a dose of 6.8-7.3 mg/kg bw The ratios of tissue:serum concentrations for tylosin measured 7-31 hours after treatment were 35.2:1 in kidney cortex, 13.9:1 in kidney medulla and 5.7:1 in liver. At 24 hours, tylosin concentrations were 35 µg/ml in bile, 13 µg/ml in urine, < 0.4 µg/ml in plasma and < 0.4 mg/kg in muscle (Nouws and Ziv, 1977b; Nouws and Ziv, 1979). When calves less than 3 weeks of age received a single intramuscular injection of tylosin base at a dose of 17.6 mg/kg bw, tylosin concentrations in lung for 24 hours after dosing ranged from 4.5 to 15.7 mg/kg, with a lung AUC_{48h}: plasma AUC_{48h} ratio of 16.6:1 (van Duyn and Folkerts, 1979). Tylosin base was administered at a dose of 10 mg/kg bw by intramuscular injection 12 hourly on three occasions to six-week old calves with pneumonia. The tylosin tissue:serum concentration ratios measured two hours after the last dose were 2.0:1 for pneumonic lung, 1.6:1 for nonpneumonic lung, 2.1:1 for liver and 2.6:1 for kidney. The highest tylosin concentration (about 3.3 mg/kg) was found in kidney, while the lowest concentration (< 0.5 mg/kg) occurred in muscle and cerebrospinal fluid (Burrows, et al., 1986).

Chickens

The distribution of tylosin in chickens has been reported and compared to other species the V_d of 0.69 l/kg in chickens is generally lower. The plasma $t_{1/2el.}$ of tylosin after single intravenous administration in healthy chickens is reportedly 0.5 hour (Kowalski, et al., 2002). When 5-7-week old chickens received 100 or 250 mg tylosin (as tartrate)/kg bw orally, maximum tylosin concentrations in urine of < 100 µg/ml at the 25 mg/kg dose, and > 1400 µg/ml at the 250 mg/kg dose, occurred 2-4 hours after dosing. Urinary concentrations of tylosin declined rapidly thereafter (WHO, 1991).

Metabolism

Rats

The metabolism of tylosin occurs primarily in the liver of rats (and other animal species). The major routes of biotransformation of tylosin are reduction, O-demethylation at the mycinose moiety, N-demethylation at the mycaminose substituent, and a combination of reduction and N-demethylation. Approximately 99% of the metabolic residues in rats was excreted in the faeces, comprising the following metabolites (expressed as a % of total ^{14}C-residues): tylosin D (10%), tylosin A (6%), and tylosin C and dihydrodesmycosin (DDM; 4%); no tylosin B was identified in the metabolic profile for rats (Table 1). Only 1% of the metabolic residues of tylosin are excreted in the urine of rats (Sieck, et al., 1978a).

In a more recent study, Fischer strain 344 rats were dosed orally by gavage with 10 mg ^{14}C-tylosin/kg bw once daily for four days (Kennington and Donoho, 1994). At four hours after the last dose, the rats were euthanized and liver and kidney were taken for assay. Liver had a mean residue of 90 μg of tylosin equivalents/kg. Analysis of an organic extract of the tissue by direct flow ionspray-mass spectrometry (ISP-MS) revealed the presence of multiple metabolites including tylosin A, tylosin D and DDM. Inconclusive evidence for the presence of cysteinyl-tylosin A residues in liver was also presented. In a separate study involving eight rats, about. 95% of the radioactivity was excreted in the faeces. The major radioactive components were tylosin D and DDM; low levels of tylosin C, the seco-acid of tylosin A, the seco-acid of tylosin D and desmethyl-dihydrodesmycosin were also present (Kennington and Donoho, 1994).

Elimination

Cattle

Tylosin excretion has also been studied following the intramuscular administration of ^{14}C-tylosin at a dose of 17.6 mg/kg bw daily for three days to two young Holstein calves weighing approximately 150kg (Kennington, et al., 1994a). Excretion in urine and faeces accounted for 48% of the administered radioactivity up to the time of slaughter (4 hours after the third and final dose). Approximately 20% of the excreted radioactivity was found in urine and 80% in faeces. Tylosin A (30%), tylosin C (25%), tylosin D (11%), and desmethyl-tylosin D (11%) were found in faecal extracts, while cysteinyl-tylosin A accounted for 70% of the total radioactivity in urine (Kennington et al., 1994a).

Tylosin is rapidly eliminated from blood in cattle with Cl_B ranging from 23.7 - 42.2 ml/min/kg in young calves and from 7.4 - 8.7 ml/min/kg in cows; $t_{1/2el.}$ of tylosin after a single intravenous administration in healthy animals ranged from 1.0 to 2.4 hours in young calves, and from 1.6 - 2.8 hours in cows. Slightly longer $t_{1/2el.}$ values of 2.2-3.2 hours were reported for tylosin after intramuscular administration (for a review, see Lewicki, 2006).

Pigs

In pigs receiving ^{14}C-labelled tylosin, 99% of the metabolic residues are excreted in faeces and 1% was excreted in urine (FAO, 1991). The principal components of the excreted residues (expressed as % of total ^{14}C-residues) were tylosin D (33%), dihydrodesmycosin (DDM; 8%) and tylosin A (6%). In addition, at least ten minor metabolites of tylosin representing 5% or less of the total residues were isolated in excreta. No tylosin B was identified in the metabolic profile (FAO, 1991).

In a GLP-compliant study, three pigs were dosed with ^{14}C-tylosin at a rate of 220 mg/kg in feed for 5 days. Approximately 94% of the radioactivity was excreted in faeces and 6% was excreted in urine (Kennington et al., 1994b). Tylosin D and dihydrodesmycosin (DDM) accounted for about 43% and 44% of the total radioactivity in faeces, respectively, from two of the pigs. Faeces from the third animal contained the seco-acid of tylosin D as the major component (approximately 56%) and tylosin D as a minor component (approximately 6%).

TISSUE RESIDUE DEPLETION STUDIES

Radio-labelled Residue Depletion Studies

Cattle

The tissue distribution and excretion of ^{14}C-tylosin has been studied in cattle following intramuscular administration (Kennington, et al., 1994a). Two Holstein calves of approximately 150 kg bw were treated once daily for three days with intramuscular injections of ^{14}C-tylosin at a dose of 17.6 mg/kg bw. Four hours after the last dose, the calves were slaughtered and tissues, bile and excreta were taken for analysis. The mean total residues of tylosin (expressed as mg of tylosin equivalents/kg [mg equiv/kg]) measured 4 hours after slaughter were 25.2 mg equiv/kg (liver), 47.8 mg equiv/kg (kidney), 2.9 mg equiv/kg (muscle), 1.5 mg equiv/kg (fat), 11.1 mg equiv/kg (lung), 2.5 mg equiv/kg (skin) and 77.2 mg equiv/kg (bile). In liver, tylosin A was the main component of the residue present. Other major metabolites in liver and kidney included tylosin D, tylosin C, and cysteinyl-tylosin A (Table 1). Lung and fat tissues were fractionated for metabolic profiling; tylosin A and cysteinyl-tylosin A were identified as the major metabolites in both tissues. By comparison, tylosin A was the only significant residue present in muscle. When these tissue samples were analyzed by HPLC with UV detection, the mean residues of tylosin A were 2.6 mg/kg (liver), 7.0mg/kg (kidney), 0.7 mg/kg (muscle) and 0.9 mg/kg (fat), which corresponded to 11%, 14%, 25% and 62% of the total residues in the respective tissues. From microbiological assay results, it was calculated that tylosin A represented 37%, 31% and 70% of the microbiologically active residues present in kidney, liver and muscle, respectively. Residues (expressed as % of total radioactivity) present in faecal extracts were tylosin A (30%), tylosin C (25%), tylosin D (11%) and desmethyl-tylosin D (11%). Urine contained cysteinyl-tylosin A as the major metabolite (69% of the total radioactivity).

Pigs

The total radioactive tissue residues that result from feeding tylosin to pigs at a feeding rate of 110 mg/kg bw twice daily were investigated (Table 1; Sieck, et al., 1978a,b). In two more recent studies, three crossbred castrated male pigs of approximately 17 kg bw were fed ^{14}C-tylosin at a dose rate of 220 mg/kg in feed for five days (Kennington, et al., 1994a). Four hours after the last dose, the pigs were slaughtered and tissues and bile were taken for assay. The mean total residues of tylosin, expressed in µg of tylosin equivalents/kg, were 450 (liver), 460 (kidney), 70 (muscle), 50 (fat), 170 (lung) and 70 (skin). Residues of tylosin A were not detected when these samples were analysed by HPLC with UV detection; the method LOQ was 50 µg/kg in all tissues. Tylosin A accounted for 12% and 8% of the total radioactive residue in liver and kidney, respectively. Smaller amounts of tylosin D (10% of the total radioactive residue in liver; 6% in kidney), DDM (5% in liver; 4% in kidney) and cysteinyl tylosin A, which readily converts to tylosin A, were also present (Table 1).

Chickens

The disposition of ^{14}C-tylosin in the edible tissues of laying hens was studied for up to 7 days after three consecutive days of *ad libitum* access to drinking water medicated with 0.53 g ^{14}C-tylosin/l (Marth, et al., 2000). Samples of liver, kidney, muscle, skin with adhering fat, and abdominal fat were collected from each of four animals sacrificed at intervals of 0, 2, 5 and 7 days after withdrawal of the medicated water. Excreta were collected daily from the group of animals sacrificed 5 days after the medicated water had been withdrawn. Total radioactive residues for tissues from two birds, one sacrificed on day zero and the other on day 5 after the medicated water was withdrawn, were 20- to 30-fold higher than for the other birds in the respective groups. The observed difference could not be attributed to a clinical or physiological abnormality. Total radioactive residues in liver for the four birds at zero withdrawal (4 hours) were 14.0, 1.0, 0.5 and 0.5 mg equivalents of ^{14}C-tylosin per kg of tissue. The mean total radioactive residue in liver declined to less than 0.1 mg of tylosin equivalents/kg by 7 days after withdrawal. By comparison, the mean total residue in kidney decreased to below 0.1 mg of tylosin equivalents/kg by 2 days after withdrawal, and in skin with adhering fat

and in abdominal fat to below 0.1 mg of tylosin equivalents/kg at all sampling times. Some 66-89% of the radioactivity in liver samples from the two high-residue birds was extractable and selected extracts were characterized by HPLC/ESI-MS-MS. Tylosin A was the principal component of the residue in liver, accounting for approximately 16% of the total residue. In excreta, radioactive residues at zero withdrawal (4 hours) ranged from 360 to 940 mg/kg and by 5 days withdrawal time, radioactive residues had declined to 11 mg/kg. Tylosin D was confirmed as the single most abundant residue at 9% of the total radioactive residue in excreta; tylosin A and the seco-acid of tylosin D were present at lower levels (Table 1). Evidence for possible N-demethylation at the mycaminose substituent was also obtained (Marth, et al., 2000).

In another study, Marth and coworkers (2001) investigated the disposition of tylosin in broiler chickens given 0.53 g ^{14}C-tylosin/l of drinking water for three days. Samples of liver, kidney, muscle, skin with adhering fat, abdominal fat and bile were collected from each of six animals at 0, 2, 5 and 7 days after withdrawal of the medicated water. The mean total radioactive residue in liver declined from 0.7 mg of tylosin equivalents/kg at day 0 to less than 0.1 mg of tylosin equivalents/kg by day 5 after withdrawal. In kidney, the mean total radioactive residue decreased to below 0.1 mg of tylosin equivalents/kg by day 5, and in muscle, skin and abdominal fat residues was < 100 μg of tylosin equivalents/kg at all time points. The liver extract contained multiple radioactive components indicating extensive metabolism; however, tylosin D was the only residue detected by HPLC/MS/MS (ESI) on account of the low residue concentrations and reduced assay sensitivity due to matrix effects. Although traces of nonpolar radioactive material were present indicating the presence of radioactivity in the tylosin A region, the radioactivity and UV signals were < LOQ of 50 μg/kg for the HPLC method. With kidney, a pooled chloroform extract was analyzed by HPLC using flow scintillation analysis; however, the quantity of radioactive residue was too low to characterize. The distribution of radioactive residues in edible tissues was in the following rank order (highest to lowest concentration): liver>kidney>skin with adhering fat> muscle.

Liver is the most appropriate target tissue because it has higher and slower depleting residues than other tissues. The data were not sufficient to define a marker residue but based on the data from other studies, the most practical marker residue is tylosin A (Marth, et al., 2000).

The data indicate that the major biotransformation products in liver are likely to result from reduction. Evidence was also found for possible demethylation and a combination of reduction and N-demethylation of tylosin. In excreta collected during the final 24 hours of dosing, tylosin A and tylosin D were the most abundant residues, accounting for 29% and 12% of the total radioactive residue, respectively. Other identified radioactive residues were each less than 10% of the total radioactive residue. These moieties included 20-dihydrodesmycosin and desmycosin. Radioactive residues in excreta accounted for at least 69% (mean) of the dose by day 7 after withdrawal of the medicated drinking water (Marth, et al., 2001).

Eggs

The distribution, metabolic fate and residue depletion of ^{14}C-tylosin in the edible tissues, eggs, and the excreta of laying hens were studied for up to 7 days withdrawal after three consecutive days of *ad libitum* access to drinking water medicated with 0.53 g ^{14}C-tylosin per litre (Burnett, et al., 1999). Eggs were collected daily from all birds throughout the dosing period and from hens after withdrawal of the medicated drinking water and prior to sacrifice. Total radioactive residues for whole eggs from 2 of the 16 treated birds at zero-day withdrawal were 1.6 to 1.7 mg equivalents tylosin A per kg of egg, while the residue ranged from 0.11 to 0.25 mg equivalents per kg for eggs collected from the remaining 14 birds. This difference was not ascribed to any clinical or physiological observation. Residues in albumen were highest in samples taken on the last day of treatment (mean of 0.4 mg equivalents of tylosin/kg) and on the following day (mean of 0.4 mg equivalents of tylosin/kg). Mean residues in albumen on day 1 and 2 after withdrawal of the medicated water were 0.16 and 0.04 mg equivalents of tylosin/kg, respectively, and were not detected (the LOD of the analytical method was 0.02 mg equivalents of tylosin/kg) in most of the eggs collected at later time points. Maximum

residues in yolk occurred in eggs collected 2 and 3 days after withdrawal of the medicated water (mean values of 0.34 and 0.34 mg equivalents of tylosin/kg, respectively) and residues in yolk declined to 0.19 and 0.07 mg equivalents of tylosin/kg at 4 and 5 days, respectively, after withdrawal of the medicated water. Residue concentrations in whole eggs were highest in eggs collected on the last day of treatment (mean of 0.33 mg equivalents of tylosin/kg) and in eggs collected on the following day (mean of 0.36 mg equivalents of tylosin/kg). Thereafter, mean residues in whole eggs depleted to 0.19, 0.13, 0.13 and 0.07 mg equivalents of tylosin/kg at 1, 2, 3 and 4 days after withdrawal of the medicated water, respectively.

In the study above, approximately 78-89% of the radioactivity from albumin and yolk samples of the two high-residue birds was extractable and selected extracts were characterized by HPLC/ESI-MS-MS. A majority of the extracted radioactivity eluted with the polar material near the reversed phase HPLC void volume, indicating a marked change in polarity from the parent compound and the presence of multiple components. Metabolites found at lower concentrations in these samples were N-desmethyl-tylosin A, dihydro-tylosin A (tylosin D), N-desmethy-dihydro-tylosin A, and O-desmethyl-tylosin A (demethylated on the mycinose moiety). The remainder of the radioactivity was predominantly polar materials, eluting early in the chromatograms. In whole eggs, tylosin A was the most abundant of the identified residues and accounted for about 17% of the total radioactive residue. Tylosin was not detected in low-residue eggs (the LOD of the analytical method was about 0.02mg/kg). This study also indicates that the primary biotransformation routes for tylosin are reduction, O-demethylation at the mycinose moiety, N-demethylation at the mycaminose substituent, and a combination of reduction and N-demethylation (Table 1). The metabolites in eggs were present at lower concentrations than the parent compound and included N-desmethyl-tylosin A, tylosin D, N-desmethyl-dihydro-tylosin A, and O-desmethyl-tylosin A (Burnett, et al., 1999).

Table 1: **Summary of studies on tylosin radiolabelled metabolism in animals**.

Animals	Source	Residue/Metabolite	References
Rats	Faeces	Tylosin D*, Tylosin A, Tylosin C, Dihydrodesmycosin (DDM)	Sieck, et al., 1978a
Rats	Faeces Liver	Tylosin D*, DDM*, Tylosin A, Tylosin C, Seco-acid of Tylosin A, Seco-acid of Tylosin D, Desmethyl-DDM Tylosin A, Tylosin D, DDM, Cysteinyl-Tylosin A	Kennington and Donoho, 1994
Pigs	Liver	DDM*, Tylosin A, 3-4 others not identified	Sieck, et al., 1978b; see also FAO (1991)
Pigs	Liver Faeces	DDM, + 3 others Tylosin D*, Tylosin A, DDM, + 10 others (each < 5%)	Sieck, et al., 1978a; see also FAO (1991)

Animals	Source	Residue/Metabolite	References
Pigs	Liver Faeces	Tylosin A, DDM, Tylosin A, Tylosin D, DDM, minor metabolites including T-1	Mertz, et al., 1982; see also FAO (1991)
Cattle	Liver/Kidney Faeces Urine	Tylosin A*, Tylosin C*, Tylosin D*, Cysteinyl-Tylosin A*, Tylosin A*, Tylosin C*, Tylosin D*, Desmethyl-Tylosin D*, Cysteinyl-Tylosin A*	Kennington, et al., 1994a
Pigs	Liver and Kidney Faeces	Tylosin A, Tylosin D, DDM, Cysteinyl-Tylosin A Seco-acid of Tylosin D*, Tylosin D	Kennington, et al., 1994b
Chickens	Eggs	Tylosin A*, N-Desmethyl-Tylosin A, Tylosin D, N-Desmethyl-dihydro-Tylosin A, Tylosin C	Burnett, et al., 1999
Chickens	Liver Excreta	Tylosin A* Dihydro-Tylosin A Tylosin A, Tylosin D, Seco-acid of Tylosin D	Marth, et al., 2000
Chickens	Liver Excreta	Tylosin D, + others not identified, Tylosin A*, Tylosin D*, 20-Dihydrodesmycosin, Tylosin B (Desmycosin)	Marth, et al., 2001
Turkeys	Liver	tylosin D (50-250 µg/kg)	Montesissa, et al., 1999
* - more than 10% of the total residue			

Residue Depletion Studies with Unlabelled Drug

Several residue studies administered different formulations of tylosin to various animal species; however, most of these studies have been previously reviewed by the Committee and are reported elsewhere (FAO, 1991). Therefore residue studies published up to 1990 have, in general, not been included in the present monograph.

Cattle

Using a crossover study design with a 21 day washout period, Tylan® 200 Injection was administered intramuscularly or subcutaneously to twelve cattle at a dose of 17.6 mg/kg bw for 5 consecutive days (Thomson and Moran, 1994). The animals were slaughtered 21 days after the last treatment and

samples of liver and kidney were collected. No residues of tylosin A in liver or kidney were detected by HPLC with UV detection (the LOD of the analytical method was 20 µg/kg).

Luperi and Villa (1999) investigated the tissue depletion of tylosin in dairy cattle. Six groups of 4 cows each received 0.05 ml/kg bw per day (10 mg tylosin/kg bw) of Tylan® 200 Injection by intramuscular administration once daily for four consecutive days. The animals were slaughtered 7, 14, 21, 28, 35 and 42 days after the last dose. Samples of kidney, liver, abdominal fat, muscle, udder and injection site tissue were collected for analyses by HPLC-UV. The LOQ for the method was reported as 50µg tylosin/kg in all tissues but it could not be confirmed based on the information provided. Tylosin residues were quantifiable in all kidney samples and in one udder sample collected 7 days after the last treatment. Residues of tylosin found at the injection sites were quantifiable in all animals sacrificed at 7 and 14 days and in two animals sacrificed 21 days after the last treatment; mean concentrations were 1620 µg/kg (day 7), 205 µg/kg (day 14) and 30 µg/kg (day 21). Tylosin concentrations were < LOQ in all other tissues from 7 days after the last treatment (Table 2).

Table 2: Group means and CV% of Tylosin residues (µg/kg) in tissues from dairy cattle dosed intramuscularly with 10mg tylosin/kg bw daily for four consecutive days.

Group	Withdrawal period	Untreated Muscle	Liver	Kidney	Abdominal fat	Injection site
1	7 days	< LOD	< LOD	73.7 (37.7)	< LOD	1620 (49.4)
2	14 days	< LOD	< LOD	7.8* (75.1)	< LOD	205 (65.8)
3	21 days	< LOD	< LOD	< LOD	< LOD	30.4 (118.2)
4	28 days	< LOD	< LOD	< LOD	< LOD	< LOD
5	35 days	< LOD	< LOD	< LOD	< LOD	< LOD
6	42 days	< LOD	< LOD	< LOD	< LOD	< LOD

Pigs

The depletion of tylosin and sulphadimidine residues was investigated following oral administration of Tylan® Sulpha Premix to pigs (Grassetti and Villa, 2001a). Four groups of pigs (2 males and 2 females per group) received the test item in feed at the nominal rate of 200 mg/kg tylosin and 200mg/kg sulphadimidine for 21 consecutive days. The mean daily dose of each active ingredient received by the four groups of pigs was 9.6, 9.8, 9.4 and 9.9 mg/kg bw per day. Animals were killed after withdrawal periods of 5, 8, 11 and 14 days and samples of kidneys, liver, muscle, and skin with adhering fat were analyzed by HPLC-UV. The LOQ of the analytical method for tylosin was 50 µg/kg for all tissues. Tylosin levels were below the limits of detection (LOD = 2.3 µg/kg for kidney, 6.0 µg/kg for liver, 4.7 µg/kg for muscle, 1.9µg/kg skin with fat, respectively) in all tissues at all time points. However, based on the information supplied the claimed values for LOQ and LOD could not be verified.

Pratts and coworkers (2002b) investigated the depletion of tylosin residues in pigs. Sixteen pigs were assigned to four groups (each n = 4) and administered tylosin base by intramuscular injection at a dose of 10 mg/kg bw once daily for 5 days. The groups of animals were sacrificed at 3, 7, 10 or 14 days after the last treatment. The highest concentration of tylosin residues was found at the injection site at 3 days (110-2500 µg/kg) and 7 days (100-4100 µg/kg) after the last treatment. Residues at the injection site depleted to below the LOQ (50 µg/kg) of the HPLC assay at 10 and 14 days after the last dose. Tylosin residues in other tissues declined at a faster rate compared to injection sites Results are summarized in Table 3.

Table 3: Tylosin residues (µg/kg) in tissues from pigs dosed intramuscularly with 10mg tylosin base/kg bw daily for five days (Prats, et al., 2002b).

Animals	Withdrawal (days)	Residue concentration (µg/kg) measured by an HPLC assay*				
		Inj. site (Muscle)	Muscle	Liver	Kidney	Skin + fat
group 1	3	440	50	70	120	84
	3	110	60	80	< 50	101
	3	1260	< 50	< 50	< 50	66
	3	2540	< 50	80	< 50	78
group 2	7	120	< 50	< 50	< 50	460
	7	310	100	< 50	110	< 50
	7	100	< 50	< 50	70	56
	7	4100	< 50	< 50	< 50	< 50
group 3	10	< 50	< 50	< 50	< 50	< 50
	10	< 50	< 50	< 50	< 50	< 50
	10	< 50	< 50	< 50	< 50	< 50
	10	< 50	< 50	< 50	< 50	< 50
group 4	14	< 50	< 50	< 50	< 50	< 50
	14	< 50	< 50	< 50	< 50	< 50
	14	< 50	< 50	< 50	< 50	< 50
	14	< 50	< 50	< 50	< 50	< 50
* HPLC assay LOQ = 50 µg/kg						

Chickens

Walker, et al. (2007) investigated the depletion of tylosin residues in muscle, liver, kidney, and skin with fat of broiler chickens following oral administration of Tylan® Soluble in drinking water at a rate of 500 mg tylosin/l. Based on water consumption and body weight data for the 5-day treatment period, the mean daily dose of tylosin was estimated at about 105 mg/kg bw per day. Groups of chickens (3 males and 3 females per group) were euthanized at 0, 12, 24 and 48 hours after withdrawal of the medicated water and samples of liver, kidney, muscle, and skin with fat were collected. The samples were analyzed for tylosin A (marker residue) using a validated HPLC-MS-MS method. The method LOQ was 50 µg/kg for all tissues. Residues of tylosin in muscle, liver, kidney and skin/fat were less than 100 µg/kg at 0 hours and approached, or were less than, 5 µg/kg (the LOD of the method) at 12 hours and 24 hours after the medicated water had been withdrawn.

Milk

In an early study, cows received intramuscular injections of tylosin at a dose of 17.6 mg/kg bw daily for five days. Tylosin residues in milk measured by a microbiological plate assay (with a sensitivity limit of 25µg/kg) at 0, 48, 72, 84 and 96 hours after the last injection were 750, 350, 140, 80 and 50µg/kg. Residues were not detected in milk samples collected at 108-144 hours following the last injection of tylosin (FAO, 1991).

A separate study reported the depletion of tylosin activity from milk after the intramuscular injection of tylosin (Matsuoka and Johnson, 1976). Five lactating cows (four cows in early lactation and one cow in late lactation) were injected once daily for 3 days at a dose of 10 mg/kg bw. Tylosin residues were below the assay sensitivity of 50 µg/kg by 48 hours after the last injection.

Another study was performed to determine the level of tylosin A residues in the milk of dairy cows following intramuscular administration of Tylan® 200 (Moran, et al., 1990). Six cows weighing 562 to 820 kg were administered 10 mg tylosin/kg bw intramuscularly for 3 days. Milk was collected twice

daily from 1 day prior to treatment to 5 days after the last treatment and analyzed for tylosin A using a validated HPLC method with ultraviolet detection. The highest concentrations of tylosin A residues were observed in milk during treatment with mean tylosin A concentrations of 1.1 mg/kg, 1.5 mg/kg and 1.4 mg/kg on days 1, 2, and 3, respectively. Concentrations of tylosin A were less than LOQ (50 µg/kg) at the afternoon milking on day 3 post-treatment and less than the LOD (20 µg/kg) at the morning milking on day 4 post-treatment.

More recently, tylosin residues were determined in the milk of 12 cows on two farms (Curtis, 1999). The cows were fed tylosin phosphate at a dose rate of 200 mg/cow/day for 17 days. Milk samples were assayed for tylosin by HPLC with UV detection (LOQ was 50 µg/kg) on days -1, 0 (initial access to medicated feed), 1, 2, 3, 4, 5, 7 and 17. Tylosin residues were not quantifiable in any milk samples.

Five high-yielding and five low-yielding dairy cows were treated intramuscularly for 5 consecutive days with Tylan® 200 at a dose of 10 mg tylosin/kg bw (Keukens, 1996). Morning and evening milk was collected from individual animals, starting at day 0 immediately prior to the first dose and continuing until 12 days after the last dose. Milk samples were analyzed for tylosin using an HPLC method with UV detection. The method LOQ and LOD were 25 µg/kg and 10 µg/kg, respectively. The maximum concentration in milk ranged from 1.3 to 2.6 mg/kg in the evening milk on day four of treatment. Tylosin residues in all samples were less than 50 µg/kg from day 3 after the last dose. There is no conclusive evidence that the marker residue was correctly quantified in this study. Dudriková and Lehotský (1998) measured tylosin residues in cows' milk by HPLC (Sokol, et al., 1996). Cows were treated with tylosin base at 10 mg/kg bw once daily for 5 days. The residues in milk are similar to that reported by Keukens (1996), and declined slowly to 30 µg/kg five days after the last treatment.

Nagy, et al. (2001) investigated tylosin residues in the milk of ewes. Tylosin was administered intramuscularly at a dose of 10 mg/kg bw once daily for 5 days. Milk residues were not detected 2 days after the last dose. Data are summarized in Table 4.

Table 4: Tylosin residues in milk (Dudriková and Lehotský, 1998; Nagy, et al., 2001).

Sampling time of experiments (hours)	Residue concentration (μg/l) measured by an HPLC assay*	
	Cows 10 mg/kg bw once daily for 5 days	Ewes 10 mg/kg bw once daily for 5 days
0◄	-	-
12	2220	630
24◄	1080	130
36	870	1822
48◄	690	470
60	1560	1650
72◄	1790	260
84	3760	1050
96◄	1650	160
108	1190	900
120(1)	1210	140
132	1010	31
144(2)	290	-
156	280	-
168(3)	160	-
180	50	-
192(4)	30	-
204	100	-
216(5)	30	-
228	30	-
240(6)	-	-
*- limit of detection = 10 μg/l; ◄- time of tylosin injection; (1-6)- days after the last injection		

Eggs

Tylosin residues in eggs and their distribution between albumen and yolk have been studied by Kan and Petz (2000). Residues in albumen reflect plasma concentrations, and the time needed to achieve a constant concentration was 2-3 days. Drug residues in yolk reflect plasma concentrations during the 10 days of rapid yolk growth. Depending on the length and timing of the exposure relative to yolk growth, residue concentrations in yolk can increase, remain constant or decrease. In general, drug residues in yolk require exposure for about 8-10 days to reach a constant concentration and depletion from yolk generally takes about 10 days (Kan and Petz, 2000). In laying hens that received tylosin at a dose of 500 g/ton of feed for 14 days, tylosin concentrations in whole eggs, measured every 2 days, reached equilibrium of 40-60μg/kg from 4-14 days after the start of treatment. Tylosin transfer rate from the diet of laying hens to eggs (i.e. the ratio of drug intake to the drug content of eggs) was only 0.005% (Furusawa, 2001). Similar transfer rates for whole eggs were reported in laying hens that had received tylosin tartrate in drinking water at a dose of 500 mg/l for 5 days (0.007%), or tylosin phosphate in feed at a dose of 400 g/ton for 7 days (0.009%) (Roudaut and Moretain, 1990).

Differences in the distribution of residue between albumen and yolk were observed in experiments comparing tylosin and other macrolide antibiotics (Roudaut and Moretain, 1990). After exposure of laying hens to tylosin tartrate in drinking water (500 mg/l for 5 days), only one hen excreted detectable residues into albumen and yolk as measured by a microbiological assay. Maximum concentrations were 0.66 mg/kg and 1.7 mg/kg in albumen and yolk, respectively. In hens receiving tylosin tartrate in drinking water (1000 mg/l for 5 days), residues of tylosin above the detection limit of the bioassay (150 μg/kg) were seen in whole eggs for up to 5 days after withdrawal of the medicated water. However, tylosin residues in albumen were detected only during the first day after

withdrawal of the medicated drinking water (Table 5). In a more recent study, where laying hens received tylosin tartrate in drinking water at a dose of 0.05%, no tylosin residues were detected in yolk samples during the 7 days on treatment or the subsequent 3 days after treatment ceased (McReynolds, et al., 2000).

Table 5: Tylosin residues (µg/kg) in eggs from hens administered tylosin tartrate in the drinking water (Roudaut and Moretain, 1990).

Days on feed	Albumen	Yolk	Whole egg
1	< 150 (ND-750)	ND	- (520)
2	190 (ND-3060)	< 200 (ND-510)	160 (0-890)
3	210 (ND-980)	< 200 (ND-1320)	200 ((0-1090)
4	250 (ND-1340)	250 (ND-1800)	250 (0-1480)
5	220 (ND-870)	400 (ND-2480)	260 (0-1350)
Days after withdrawal			
1	230 (ND-830)	650 (ND-2990)	370 (0-1460)
2	ND	470 (ND-2390)	140 (0-710)
3	-	390 (ND-2000)	130 (0-630)
4	-	< 200 (ND-1280)	80 (0-423)
5	-	< 200 (ND-660)	- (190)
6	-	< 200 (ND-230)	-
7	-	ND	-
Limit of detection of microbiological assay = 150 µg/kg (albumen) and 200 µg/kg (yolks); ND - not detected; data in parentheses = analytical range			

The residue depletion profiles of tylosin in eggs were investigated in laying hens after oral administration of Tylan® G250 in the diet (Grassetti and Villa, 2001b). Twenty-four laying hens received tylosin phosphate at an inclusion rate of 800 mg/kg in feed for 5 consecutive days. Tylosin residue levels were determined in eggs produced the day before dosing to 5 days after dosing ceased. The HPLC method with UV detection was not specific for tylosin A; the LOQ was 50 µg/kg and the LOD was 13 µg/kg for tylosin residues. One egg collected on the fifth day of dosing contained a residue of 75 µg tylosin/kg. Tylosin residues in all other eggs were less than the LOQ of the method.

In a separate study, eggs were collected daily from seventeen chickens offered water medicated with Tylan® Soluble to provide 500 mg tylosin activity/l for 3 days (Warren, 1998). Tylosin residues in 12 eggs selected at random each day were determined by HPLC with UV detection; the method was not specific for tylosin A. During the treatment period, only 4 of 36 eggs collected during the treatment period contained residues exceeding the LOQ (50 µg tylosin/kg) of the method. After withdrawal of the medicated water, no residue concentrations exceeded the LOQ and the majority of eggs contained residues below the LOD (10 µg tylosin/kg).

King and Walker (2007) investigated the depletion of tylosin residues in eggs following the administration of drinking water medicated with Tylan Soluble at a concentration of 500 mg tylosin/l for 5 consecutive days to twenty-two laying hens. This study was designed to meet the requirements of the EMEA/CVMP Guideline 036/95: “Note for guidance: Approach towards harmonisation of withdrawal periods” (EMEA, 1996). The mean daily dose of tylosin calculated from water consumption and body weight data during the 5-day treatment period was about 92 mg tylosin/kg bw (dose range was 86.9 to 96.7 mg tylosin/kg bw). Eggs were collected daily from each bird and individually homogenised prior to analysis for marker residue (tylosin A) using a validated HPLC method (Adam, et al., 2007). The mean concentration of tylosin was < LOQ on all days. The highest concentration of tylosin was 117 µg/kg in an egg at day 2 after initiation of treatment; the concentration of tylosin was below LOQ after day 6. Two reports have been published that describe findings consistent with those of King and Walker (Furusawa, 2001; Hamscher, et al., 2006). In these studies, hens were medicated with feed containing 500 mg/kg or 1500 mg/kg of tylosin. Residues in

eggs determined by HPLC and HPLC-ESI-MS-MS were near or above the LOD (60µg/kg) during treatment, declining immediately when the medicated feed was withdrawn (Hamscher, et al., 2006).

Honey

Information in the scientific literature concerning residues of tylosin in honey is scant. Feldlaufer and coworkers (2006) reported residue depletion studies conducted in the USA which determined the incurred residues of tylosin in honey resulting when tylosin tartrate was applied as a dust in confectioner's sugar to honeybee colonies. Each colony comprised approximately 40,000 worker bees. In order to maximize the likelihood of residues being detected, tylosin treatments were applied during the honey flow. One hive was an untreated control; two hives were treated on three occasions with 200 mg or 1000 mg of tylosin (total of 600 mg and 3000 mg of tylosin) over a two-week period. Honey was sampled during and after tylosin administration. Tylosin residues in honey from brood chambers and supers were measured by microbiological assay. Results are shown in Table 6. Honey samples collected from supers following the treatment of colonies with the target therapeutic dose of 600 mg of tylosin/hive contained 160 µg of tylosin equivalents/kg of honey at 3 weeks after the last treatment.

Table 6: Mean concentrations (mg/kg) of tylosin in brood chamber and surplus honey (Feldlaufer, et al., 2006).

Treatment (mg/hive)	0 day (on treatment)	7 days after final treatment	14 days after final treatment	21 days after final treatment
Brood chamber				
0	-	0.12 (0.03, 0.3*)	0.0 (0.0, 0.06*)	0.0 (0.0, 0.03*)
600	-	1. 5 (0.7, 3.5*)	0.5 (0.2, 1.0*)	0.4 (0.2, 0.9*)
3000	-	5.6 (2.2, 17.5*)	4.5 (1.9, 13.4*)	2.0 (0.9, 4.9*)
Super				
0	0.05 (0.0, 0.2*)	0.0 (0.0, 0.06*)	0.0 (0.0, 0.07*)	0.05 (0.0, 0.2*)
600	1.3 (0.6, 3.1*)	0.4 (0.2, 0.9*)	0.3 (0.1, 0.7*)	0.2 (0.05, 0.4*)
3000	8.7 (3.2, 34.3*)	3.6 (1.5, 9.9*)	2.5 (1.1, 6.3*)	1.6 (0.7, 3.9*)
* (lower, upper 95% confidence limits- rounded values)				

Nalda and coworkers (2006) reported a study in honey which investigated residues of tylosin A, B, C and D. This trial was conducted in Spain and samples were collected in spring when the honey flow is adequate for attaining high residues in honey. Fifteen beehives with comparable bee populations and health status were selected and assigned to three groups. One group was fed a placebo; a second group was administered a sugar mixture containing 200 mg/kg of tylosin (identified in the study as 201 to 205); and a third group was administered a sugar mixture containing 400 mg/kg of tylosin (identified in the study as 401 to 405). One month after the sugar mixture had been consumed, honey was collected from the brood chambers and analysed with a validated HPLC-ESI-MS-MS method. Honey from beehives treated with tylosin contained residues of tylosin A, B, C and D with tylosin A accounting for more than 80% of the total residue. Residues of tylosin in honey were not correlated to the applied dose. For example, honey from a hive treated with 200mg/kg had higher concentrations of residues than some beehives treated with 400 mg/kg of tylosin. This could be attributed to the different social behaviour of beehives, food storage, etc. The data demonstrate that residues of tylosin B, which accounts for approximately 6-12% of the total residue, and tylosin C and tylosin D collectively account for approximately 15% of tylosin residues in honey. Nalda and coworkers (2006) suggested that further field experiments are necessary to optimise the dosage such that residues of tylosin in honey may be reduced.

Table 7: Residues (µg/kg) of tylosin A, B, C and D in honey samples from treated beehives (Nalda, et al., 2006).

Sample	Tylosin A (TA)	Tylosin B (TB)	TA:TB ratio	Tylosin C (TC)	Tylosin D (TD)	Total (%TA)
Placebo	< LOD	< LOD	-	< LOD	< LOD	< LOD
200 mg of tylosin per kg of sugar mixture *						
201	1230	90	13.7	< LOD	110	1430 (86)
202	1030	100	10.3	< LOD	110	1240 (83)
203	600	70	8.6	< LOD	20	690 (87)
204	870280	16	54.4	< LOD	30	1060 (82)
205		410	10.4	70	180	4940 (87)
400 mg of tylosin per kg of sugar mixture *						
401	1550	230	6.7	10	80	1870 (83)
402	3740	310	12.1	20	140	4210 (89)
403	500	70	7.1	< LOD	10	580 (86)
404	2110	330	6.4	20	90	2550 (83)
405	5730	700	8.2	80	210	6720 (85)
Limit of detection (LOD) for the HPLC-ESI-MS method: 2 (TA), 3 (TB), 2 (TC) and 2 (TD) µg/kg; * - no information was provided regarding the length of time that had elapsed between the application of the tylosin formulation and the sampling time						

In a Canadian experiment, Thompson and coworkers (2007) used a slightly modified version of a previously reported HPLC-ESI-MS-MS method (Thompson, et al., 2005) for determining tylosin A and B in honey. The hives in the study were healthy, single brood chamber colonies containing approximately 30,000 adult honeybees. Twenty colonies with similar populations of brood and adult bees were identified and randomly assigned to five treatment groups. Treatments contained varying amounts of tylosin tartrate as Tylan® Soluble 100GM in two different formulations. One formulation consisted of either 0 mg or 300 mg of tylosin tartrate mixed with 20 g of confectioner's sugar. The second formulation consisted of the antibiotic incorporated into a 100g pollen patty. Each patty consisted of 40% milled pollen, 20% soy flour and 40% (v/v) sucrose syrup, mixed into a moist kneadable texture, to which 300, 900 or 1500 mg tylosin tartrate was added. During September 2004, treatments were applied to the top bars of the brood chambers on three occasions at weekly intervals. Colonies in the sugar dusting treatments received, in total, either 0 mg or 900 mg of tylosin tartrate whereas colonies treated with pollen patties received, in total, 900, 2700 or 4500 mg of tylosin tartrate. These treatment rates exceeded the hypothesized total target dose of 600 mg tylosin tartrate per colony and were chosen to examine the concentration of residue carried over to the following year. For residue determination, 15 g samples of newly deposited honey were collected from colonies in July 2005, approximately 1 week after the start of the summer honey flow. Samples were stored at -20°C prior to analysis (Thompson, et al., 2007).

Table 8: Residues (µg/kg) of tylosin A and tylosin B in incurred honey after 294 days of withdrawal [a] (Thompson, et al., 2007).

Replicate	Source [b]	Tylosin A (TA)	Tylosin B (TB)	TA:TB ratio
Sugar dust: 900 mg of tylosin per colony				
1	Brood chamber	114	97	1.2
2		62	44	1.4
3		11	10	1.1
4		ND [c]	ND	-
1	Super	179	150	1.2
2		46	31	1.2
3		32	32	1.0
4		ND	ND	-
Pollen patty [d]: 2700 mg of tylosin per colony				
1	Brood chamber	19	22	0.9
2		80	60	1.3
3		16	13	1.2
4		28	24	1.2
1	Super	29	33	0.9
2		64	48	1.3
3		ND	ND	-
4		ND	ND	-
Pollen patty [d]: 4500 mg of tylosin per colony				
1	Brood chamber	77	60	1.3
2		23	14	1.6
3		16	13	1.2
4		16	17	0.9
1	Super	ND	ND	-
2		ND	ND	-
3		23	19	1.2
4		6	7	0.9

[a] – colonies were treated on three successive occasions, 7 days apart, during September 2004 and were sampled in July 2005; withdrawal period is calculated from date of last application to sample collection;
[b] – honey samples from brood chambers and supers were from the same colonies within treatments and replicates; supers contain honey normally extracted for human consumption;
[c] – ND, non-detectable (limit of detection of HPLC-ESI-MS-MS method: 0.4 µg/kg (tylosin A) and 1.1 µg/kg (tylosin B); practical limit of quantitation: 5 µg/kg (tylosin A); 5 µg/kg (tylosin B);
[d] – values for colonies treated with a total of 900 mg of tylosin tartrate formulated in pollen patties are not listed because no residues were detected in honey taken from brood chambers or supers

Tylosin A degrades to tylosin B in an acidic medium such as honey and studies into the stability of tylosin residues in honey during storage have been performed using HPLC. Kochansky (2004) reported the conversion of tylosin to tylosin B with a half-life of approximately 4 months during storage at 34°C. The stability of tylosin A in honey matrices has also been investigated by spiking a series of replicate honey samples with tylosin A and storing them in the dark at -20°C and 20°C. Samples were analyzed at 2-weekly intervals for a period of 16 weeks; no appreciable degradation of tylosin A was observed when stored at -20°C. Over the same period of time, approximately 20% of tylosin A degraded to tylosin B when stored at ambient temperature (Thompson, et al., 2007).

Honey samples drawn from bee colonies treated with a commercial formulation of tylosin were analyzed for the presence of both tylosin A and tylosin B. Though the formulation of tylosin in sugar dustings greatly increased the propensity and concentration of tylosin A and tylosin B within incurred honey samples, a relatively consistent ratio (from 0.9 to 1.6) of tylosin A to tylosin B was observed

across all samples irrespective of treatment and source of honey. Accordingly, for samples with detectable residues, the ratio of tylosin A to tylosin B was similar, with an overall average 1.2:1 (Table 8). This suggests that after a prolonged withdrawal period (294 days in this study), the contributions of tylosin A and its primary breakdown product, tylosin B, are of comparable importance in terms of antimicrobial load (Thompson, et al., 2007).

METHODS OF ANALYSIS

A validated analytical method for the quantitation of the marker residue in target animal tissues is necessary for enforcement of MRLs and is required as part of the information for the evaluation of veterinary drug residues (FAO, 2000). Many different analytical methods (screening or confirmatory) have been described for tylosin and other macrolide antibiotics in the open literature between 1985 and 2005. These methods are reportedly suitable for quantifying tylosin and/or its degradants and metabolites in aqueous solutions and fermentation media, animal feeds, environmental samples and excreta. A number of methods for the detection of tylosin or other macrolides in biological fluids and animal tissues have also been published. Microbiological assays that lack specificity and are not suitable for identifying the exact nature of an antibiotic residue, are commonly used for screening samples for tylosin residues. More specific methods, such as liquid chromatography coupled with ultraviolet (LC-UV) detection, have been proposed for the determination of tylosin residues in animal tissues. Gas chromatography coupled to mass spectrometry (GC-MS) has been described as a confirmatory method for tylosin residue analysis. Several other methods based on a combination of liquid chromatography with mass spectrometry (HPLC-MS) and tandem mass spectrometry (HPLC/MS/MS) have been reported for quantitation and confirmation of tylosin residues in animal tissues.

Apart from clearly described liquid scintillation counting methods used in ^{14}C-tylosin residue studies (Kennigton and Donoho, 1994; Kennington et al., 1994a; Kennington et al., 1994b; Burnett et al., 1999; Marth et al., 2000; Marth et al., 2001), several HPLC or HPLC/MS/MS methods for tylosin A (or other tylosin factors/metabolites) residue analysis were provided for evaluation by the Committee. Only those which are validated are discussed below.

An analytical method was provided for determining tylosin A in chicken whole eggs (Adam, et al., 2007). The analytical method includes homogenization with methanol/acetonitrile/0.1 M ascorbic acid followed by centrifugation. Tylosin is then isolated from the supernatant using C18 Solid Phase Extraction (SPE). The purified sample is evaporated to dryness and reconstituted for further HPLC separation on a phenyl stationary phase, and UV detection at 280 nm. Quantification of tylosin is as factor A. Analytical recoveries ranged from 74–87% with coefficients of variation of 3.6–9.6%. Intra-day and inter-day accuracy at MRL level was 78–80% and precision (intra-day and inter-day) in the 5–9% range. The method LOQ for tylosin A was claimed to be 50µg/kg for whole eggs; however, critical analysis of the information provided suggested the LOQ is likely to be above 100 µg/kg. Similarly, the LOD for tylosin A was claimed to be 4 µg/kg for whole eggs but on the basis of the information provided, the LOD is likely to approximate 50 µg/kg. The method is not acceptable for measuring tylosin residues at or below a concentration of 100 µg/kg but is acceptable for measuring higher concentrations.

A validated HPLC/MS/MS method with electrospray ionization is available for determining residues of tylosin A in the edible tissues of chickens and in eggs (Roberts, 2007). The analytical method involves extraction from tissue and eggs by homogenizing with acidified acetonitrile followed by centrifugation. The supernatant is diluted with acetonitrile/water and then analysed by HPLC with detection by tandem mass spectrometry (HPLC/MS/MS) operating in the selected reaction monitoring (SRM) mode. Acceptable specificity, sensitivity, linearity, precision, recovery and accuracy were demonstrated for the method. Analytical recoveries ranged from 85 to 103% with coefficients of variation of 5–10%. The method LOQ was 50 µg/kg for liver, kidney, muscle and skin with fat and 100 µg/kg for eggs. The LOD of the analytical method was 5 µg/kg for all tissues and eggs. The ion chromatograms and other information provided confirmed the claimed performance characteristics.

Intra-day and inter-day accuracy at a potential MRL level was in the 85–102% range, and intra-day and inter-day precision was in the 5–10% range. The assay specificity was acceptable as attested by the quality of the signals on the ion chromatograms provided. Assay linearity was shown to be acceptable in the fortified matrix range of 10 to 500 µg/kg after extraction and dilution. Although there were no significant matrix effects in any matrix, the inclusion of an internal standard would correct for any matrix effect during the electrospray ionization process. This method could be extended to other matrices and is a suitable analytical method for regulatory use with residues in the edible tissues of cattle, pigs, chickens, milk and eggs.

A microbiological assay was provided for analysis of edible tissues of cattle and pigs; however, the method was not appropriately validated. In honey, a validated method is required for the analysis of residues of tylosin A plus tylosin B (see section Appraisal below); however, a suitable method was not available for review.

New methods for tylosin analysis have appeared recently in the open literature. These include methods for detecting tylosin and/or its metabolites and degradation products in aqueous solutions (Song, et al., 2007; Hu, et al., 2008) and animal feeds (Peng and Bang-Ce, 2006; González de la Huebra, et al., 2007; Vincent, et al., 2007). New methods for the detection of tylosin and/or other macrolides in biological fluids and animal tissues have also been published (García-Mayor, et al., 2006; Hamscher, et al., 2006; Tang, et al., 2006; Wang, et al., 2006; Litterio, et al., 2007) (Table 9).

Table 9: Overview of the newest HPLC or HPLC/MS/MS methods for residues of tylosin in foods of animal origin.

Method of detection	Matrix	Compounds detected	LOD [1,2] (µg/kg)	Reported Validation Status [3]	Reference
HPLC-UV, PDA	Sheep: Milk	Tylosin Erythromycin Oleandomycin Roxithromycin Josamycin Spiramycin Ivermectin	24.1 [4]	Yes	García-Mayor, et al., 2006
HPLC-ESI-MS-MS	Bovine: Milk	Tylosin Spiramycin Tilmicosin Oleandomycin Erythromycin	0.06	Yes	Wang, et al., 2006
HPLC-ESI-MS-MS	Laying hens: Eggs	Tylosin	1 [4]	Yes	Hamscher, et al., 2006
HPLC-ESI-MS-MS screening method	Animal muscle	Tylosin A + 4 macrolides + 6 fluoroquinolones + 3 other	0.1	Yes (only for screening)	Tang,et al.,2006

ESI - electrospray ionisation;
[1] - limit of detection (LOD);
[2] - for multiresidue methods only a value for tylosin was specified;
[3] - declared by authors for the time of publication;
[4] - limit of quantitation (LOQ)

Several analytical methods (including optical SPR biosensor screening assay) concerning tylosin residues in honey were presented (Thompson, et al., 2003; Benetti, et al., 2004; Wang, 2004; Caldow, et al., 2005; Thompson, et al., 2005; Nalda, et al., 2006; Thompson, et al., 2007; Hammel, et al., 2008) (Table 10).

Table 10: Overview of the HPLC/MS or HPLC/MS/MS methods for residues of tylosin in honey.

Method of detection	Compounds detected	LOD [a,b] (µg/kg)	Reported Validation Status [c]	Reference
HPLC-API-MS	Tylosin Lincomycin	10	Yes	Thompson, et al., 2003
HPLC-ESI-MS-MS	Tylosin	Not specified Ccα = 2.6 µg/kg [d] Ccβ = 4.4 µg/kg [d]	Yes	Benetti, et al.,2004
HPLC-ESI-MS-MS	Tylosin Spiramycin Tilmicosin Oleandomycin Erythromycin	0.01 LOC = 0.4 µg/kg [e]	Yes	Wang, 2004
HPLC-ESI-MS-MS	Tylosin Lincomycin	2	Yes	Thompson, et al., 2005
HPLC-PDA-ESI-MS	Tylosin A Tylosin B Tylosin C Tylosin D	2 3 2 2	Yes	Nalda, et al.,2006
HPLC-ESI-MS-MS	Tylosin A Tylosin B	0.4 1.1	Yes	Thompson et al., 2007
HPLC-ESI-MS-MS screening method	Tylosin + 24 antibiotics + 17 sulphonamides	48	Yes (only for screening)	Hammel, et al.,2008

API - atmospheric pressure ionisation; ESI - electrospray ionisation;
[a] - limit of detection (LOD);
[b] - for multiresidue methods only a value for tylosin was specified;
[c] - declared by authors for the time of publication;
[d] – in the 2002/657/EC European decision Ccα and Ccβ replace the LOD and LOQ;
[e] - limit of confirmation (LOC)

APPRAISAL

Tylosin is an old drug with a long history of use. It was first evaluated at the twelfth meeting of the Committee in 1968 when it was concluded that tylosin used in animal feed or in veterinary medicine should not give rise to detectable residues in edible products of animal origin. No ADI was established. The drug was subsequently evaluated at the thirty-eighth meeting of the Committee. At that meeting, the Committee was not able to establish an ADI due to deficiencies in the toxicological and microbiological data submitted. Information addressing the deficiencies identified by the thirty-eighth meeting of the Committee was requested for evaluation by the sixty-sixth meeting of the Committee but none was provided. New data addressing the deficiencies were made available for the present meeting of the Committee to evaluate.

Tylosin is a macrolide antibiotic produced by fermentation from a strain of the soil microorganism, *Streptomyces fradiae*. It is a mixture of four compounds. The main product is tylosin A (> 80%) and the minor components are tylosin B, C and D, which may be present in varying amounts. It is active against Gram-positive bacteria, Mycoplasma and certain Gram-negative bacteria. Tylosin and its phosphate and tartrate salts are registered exclusively for veterinary use in several countries. Tylosin is used primarily in the chronic respiratory disease (CRD) complex in chickens and infectious sinusitis in turkeys caused by *Mycoplasma gallisepticum*. It is also used to treat swine and bovine respiratory diseases and swine dysentery, and other infections caused by organisms sensitive to tylosin.

Tylosin is a highly lipid soluble, weak organic base (pKa = 7.73) that readily forms salts and esters. It is slightly to moderately bound to plasma proteins (30-47%). Tylosin is widely distributed in body fluids and tissues with a V_d which ranges from 1–14.6 l/kg in different animal species. From a residue perspective, the distribution of tylosin is highly dependent on the route of administration. When administered by injection, tylosin residues are generally highest and most persistent in kidney with the exception of injection site residues. By contrast, residue concentrations following oral administration are generally higher in liver than in other tissues. The concentration of tylosin residues observed after oral administration is generally lower than after injectable administration.

The biotransformation of tylosin has been studied in rats, chickens, pigs and cattle and the comparative metabolism was shown to be qualitatively similar for these species. Tylosin is principally metabolized in the liver resulting in four major metabolites and several minor metabolites in most species. The primary biotransformation routes for tylosin are reduction, O-demethylation at the mycinose moiety, N-demethylation at the mycaminose substituent and a combination of reduction and N-demethylation. Tylosin A is the most abundant residue in rats, chickens, pigs and cattle while a major metabolic pathway is the reduction of tylosin A to tylosin D.

A radiometric study was available in calves treated intramuscularly daily with ^{14}C-tylosin/kg. Tylosin A accounted for approximately 11% (liver), 15% (kidney), 25% (muscle) and 62% (fat) of the total residues and represented 31% (liver), 37% (kidney) and 70% (muscle) of the microbiologically active residues present. Residues of tylosin A in liver and kidney were less than 20 µg/kg in cattle receiving the same dose of unlabelled tylosin/kg daily for five consecutive days intramuscularly or subcutaneously at 21 days after the last dose.

A radiometric study was conducted in pigs using ^{14}C-tylosin in feed at an inclusion rate of 220 mg/kg. Tylosin A accounted for 12.3% of the total residues in liver and 7.6% in kidney. The mean total residues of tylosin (µg of tylosin equivalents/kg) were 450, 460, 70, 50 and 70 in liver, kidney, muscle, fat and skin, respectively. Tylosin A was not detected in any sample (LOQ was 50 µg/kg).

Residue depletion studies in cattle and pigs indicated that tissue residues of tylosin were generally low to non-detectable following the oral route of administration and depleted rapidly with predictable kinetics following intramuscular injection. High concentrations of tylosin residues were found at the injection sites in both dairy cattle and pigs. Residues at the injection site depleted to below the LOQ (50 µg/kg) by 10 days after the last dose.

Radiometric studies were conducted in laying hens and broiler chickens. In laying hens, total radioactive residues in liver at zero day withdrawal were 13.7, 1.0, 0.5 and 0.5 mg equivalents of ^{14}C-tylosin/kg. The mean total radioactive residue in liver declined to less than 0.1 mg of tylosin equivalents/kg by 7 days after withdrawal and in kidney decreased to below 0.1 mg of tylosin equivalents/kg by 2 days after withdrawal. Residues in skin with adhering fat and in abdominal fat were below 0.1 mg of tylosin equivalents/kg at zero day withdrawal. In broilers, the mean total radioactive residue in liver declined from 0.7 mg of tylosin equivalents/kg at zero day withdrawal to less than 0.1 mg of tylosin equivalents/kg by 5 days withdrawal; in kidney, to less than 100 µg of tylosin equivalents/kg by 5 days withdrawal; in muscle, skin with adhering fat and abdominal fat to less than 100 µg of tylosin equivalents/kg at zero day withdrawal. In a residue depletion study broiler

chickens receiving tylosin in drinking water for five days, the residues of tylosin A in liver, kidney, muscle and skin with adhering fat were less than 100 μg/kg at 0 hours and at or below 5 μg/kg at 12 hours after the medicated water had been withdrawn.

Residue depletion studies were performed in cows' milk. When tylosin phosphate was included in the feed at a rate of 200 mg/cow/day, tylosin residues were not quantifiable (LOQ 50 μg/kg) in any milk samples collected during treatment. In a second study cows were treated intramuscularly with 10mg tylosin/kg bw for 5 days. The maximum concentration in milk was 1.3 to 2.6 mg/kg on the fourth day of treatment. Concentration of tylosin residues in all samples was less than 50 μg/kg from day 3 after the last dose. In a third study, dairy cows were administered the same intramuscular dose for 3 days. The highest concentrations of tylosin A residues were observed in milk during treatment with mean tylosin A concentrations of 1.1 -1.5 mg/kg on days 1, 2 and 3. Concentrations of tylosin A were less than the LOQ (50 μg/kg) at the afternoon milking on day 3 post-treatment and less than the LOD (20 μg/kg) at day 4 post-treatment.

One radiometric study and three depletion studies with unlabelled tylosin were performed in eggs. In the radiolabelled study, variable results were obtained. The residues in whole eggs were 108 – 245 μg tylosin equivalents/kg in 14 of 16 birds but negligible residues in the other 2. Residue concentrations in whole eggs were highest in eggs collected on the last day of treatment. Mean residues in whole eggs depleted over four days after withdrawal of the medicated water. Tylosin A was the most abundant of the residues in whole eggs and at the highest concentration of tylosin equivalents, and accounted for approximately 17% of the total radioactive residues. Tylosin A was not detected in eggs produced by the other 14 birds (LOD 20 μg/kg). In a residue depletion study with unlabelled drug at an inclusion rate of 800mg/kg in feed for 5 consecutive days only one egg collected on the fifth day of dosing contained a measurable residue of 75 μg tylosin/kg. The concentration of residues in all other eggs was less than the method LOQ (50 μg/kg). In residue depletion studies with unlabelled drug in the drinking water of laying hens with 500 mg tylosin activity per litre for 3 or 5 days, only 4 of 36 eggs collected during the treatment period contained residues above the LOQ (50 μg tylosin/kg). After withdrawal of the medicated water, no residue concentrations exceeded the LOQ with the majority of eggs having residues below the LOD (10 μg/kg). In the last study, the mean concentration of tylosin A in whole eggs was less than the LOQ of the method (50 μg/kg).

Tylosin A was identified as the marker residue for tylosin in the tissues of chickens, pigs and cattle as well as in milk and eggs. Tylosin A represents the most significant residue and corresponds to the major microbiologically active residue of concern. A validated HPLC/MS/MS method with electrospray ionization is available for determining residues of tylosin A in the edible tissues of chickens and eggs, and could be extended to other matrices. This method is suitable for regulatory use to detect and quantify residues of tylosin A.

As distinct from mammalian and avian tissues, tylosin B is a major end product in honey resulting from the conversion of tylosin A to tylosin B in acidic media such as honey. The conversion accounts for the ratio of tylosin A concentration/tylosin B concentration varying as a function of time. Tylosin B contributes significantly to the antimicrobial activity of tylosin residues in honey, requiring that both tylosin A and tylosin B are taken into account when considering dietary intake of residues. This implies that tylosin A is not a suitable marker for residues of tylosin in honey, unlike the situation with chickens, pigs, cattle, milk and eggs. In the absence of a suitably validated method for quantifying the microbiological activity of residues of tylosin A and tylosin B in honey, it is not appropriate to recommend a MRL for tylosin in honey.

MAXIMUM RESIDUE LIMITS

In recommending MRLs, the Committee took into account the following factors:

- An ADI of 0-30 μg/kg bw based on a microbiological endpoint was established by the seventieth meeting of the Committee, equivalent to 0-1800 μg for a 60-kg person.

- The marker residue is tylosin A and represents approximately 100% of the microbiologically active residues, except in honey. This information is incorporated in the calculation of the intake estimates to ensure that they correctly reflect residues of microbiological concern.
- Liver and muscle are suitable target tissues.
- A validated analytical method is available for analysis of tylosin A residues in edible tissues of chickens and in eggs, and could be extended to the edible tissues of cattle and pigs and to milk.
- The MRLs for all edible tissues of cattle, pigs and chickens were based on the data provided.
- The MRL for eggs was based on the highest value of tylosin A concentration observed.
- The MRL for milk was based on twice the LOQ.

On the basis of the above considerations, the Committee recommended the following MRLs for edible tissues of cattle, pigs and chickens, expressed as the marker residue, tylosin A: muscle, 100 µg/kg; liver, 100 µg/kg; kidney, 100 µg/kg; fat, 100 µg/kg (cattle and pigs); and skin/fat, 100 µg/kg (chickens). The Committee also recommended a MRL for milk of 100 µg/kg and a MRL for eggs of 300 µg/kg, both expressed as the marker residue, tylosin A.

ESTIMATION OF DAILY INTAKE

The sixty-sixth meeting of the Committee agreed to apply a new approach to estimate chronic exposure to residues of veterinary drugs in food. However, the Estimated Daily Intake (EDI) for tylosin A was not estimated because there were insufficient quantitative data points to calculate the median values for residues in food animal tissues. Using the model diet and the microbiological activity of tylosin A as 100% of the microbiological activity of the residue, the recommended MRLs would result in an intake of 230 µg, which represents 13% of the upper bound of the ADI (1800 µg for a 60-kg person) (Table 11).

Table 11: Estimation of daily intake of tylosin A residues.

Tissue	MRL (µg/kg)	Standard food basket (kg)	Microbiological Activity[2]	Daily intake (µg)
Muscle	100	0.3	100%	30
Liver	100	0.1	100%	10
Kidney	100	0.05	100%	5
Fat[1]	100	0.05	100%	5
Milk	100	1.5	100%	150
Eggs	300	0.1	100%	30
1. In chickens: skin/fat. 2. Microbiological activity of Tylosin A			Total daily intake	230
			Upper limit of the microbiological ADI	1800
			% of ADI	12.8

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ANNEX 1

RECOMMENDATIONS ON COMPOUNDS ON THE AGENDA AND FURTHER INFORMATION REQUIRED

Avilamycin (antimicrobial agent)

Acceptable daily intake: The Committee established an ADI of 0–2 mg/kg bw on the basis of a NOAEL of 150 mg avilamycin activity/kg bw per day and a safety factor of 100 and rounding to one significant figure.

Residue definition: Dichloroisoeverninic acid (DIA)

Recommended maximum residue limits (MRLs)

Species	Skin/fat (µg/kg)	Kidney (µg/kg)	Liver (µg/kg)	Muscle (µg/kg)
Pigs	200	200	300	200
Chickens	200	200	300	200
Turkeys	200	200	300	200
Rabbits	200	200	300	200

Dexamethasone (glucocorticosteroid)

Acceptable daily intake: The Committee established an ADI of 0–0.015 µg/kg bw at the 42nd meeting of the Committee (WHO TRS No. 851, 1995, reference 9).

Residue definition: Dexamethasone

Recommended maximum residue limits (MRLs)

Species	Kidney (µg/kg)	Liver (µg/kg)	Muscle (µg/kg)	Milk (µg/l)
Cattle	1.0	2.0	1.0	0.3
Pigs	1.0	2.0	1.0	
Horses	1.0	2.0	1.0	

Malachite green (antimicrobial agent and contaminant)

Acceptable daily intake: The Committee considered it inappropriate to establish an ADI for malachite green and did not support the use of malachite green for food-producing animals.

Residues: The Committee did not recommend MRLs for malachite green and leucomalachite green, as it did not support the use of malachite green for food-producing animals.

Melengestrol acetate (production aid)

Acceptable daily intake: The Committee established an ADI of 0–0.03 µg/kg bw at its 54th meeting (WHO TRS No. 900, 2001, reference 18). It did not consider it necessary to reconsider the ADI at the current meeting on the basis of new data provided.

Residues: The MRLs that were recommended by the 66th meeting of the Committee (WHO TRS No. 939, 2006, reference 22) were not reconsidered and were maintained.

Monensin (antimicrobial agent and production aid)

Acceptable daily intake: The Committee established an ADI of 0–10 µg/kg bw on the basis of a NOAEL of 1.14 mg/kg bw per day and a safety factor of 100 and rounding to one significant figure.

Residue definition: Monensin

Recommended maximum residue limits (MRLs)

Species	Fat (µg/kg)	Kidney (µg/kg)	Liver (µg/kg)	Muscle (µg/kg)	Milk (µg/kg)
Cattle	100	10	20	10	2
Sheep	100	10	20	10	
Goats	100	10	20	10	
Chickens	100	10	10	10	
Turkeys	100	10	10	10	
Quail	100	10	10	10	

Narasin (antimicrobial agent and production aid)

Acceptable daily intake: The Committee established an ADI of 0–5 µg/kg bw on the basis of a NOAEL of 0.5 mg/kg bw per day and a safety factor of 100.

Residues: Narasin A

Recommended maximum residue limits (MRLs)

Species	Fat (µg/kg)	Kidney (µg/kg)	Liver (µg/kg)	Muscle (µg/kg)
Cattle	50[a]	15[a]	50[a]	15[a]
Chickens	50	15	50	15
Pigs	50	15	50	15

[a] The MRL is temporary.

Before a re-evaluation of narasin with the aim of recommending MRLs in tissues of cattle, the Committee would require a detailed description of a regulatory method, including its performance characteristics and validation data. This information is required by the end of 2010.

Tilmicosin (antimicrobial agent)

Acceptable daily intake: The Committee established an ADI of 0–40 µg/kg bw at its 47th meeting (WHO TRS No. 876, 1998, reference 12).

Residue definition: Tilmicosin

Recommended maximum residue limits (MRLs)

Species	Skin/fat (µg/kg)	Kidney (µg/kg)	Liver (µg/kg)	Muscle (µg/kg)
Chickens	250	600	2400	150
Turkeys	250	1200	1400	100

The Committee was not able to recommend a MRL for sheep milk.

Before a re-evaluation of tilmicosin with the aim of recommending MRLs in tissues of rabbits, the Committee would require adequately designed residue studies with doses and routes of administration under authorized conditions of use and using a validated method suitable for the purpose.

Triclabendazole (anthelminthic)

Acceptable daily intake: The Committee established an ADI of 0–3 µg/kg bw at its 40th meeting (WHO TRS No. 832, 1993, reference 8).

Residue definition: Ketotriclabendazole

Recommended maximum residue limits (MRLs)

Species	Fat (µg/kg)	Kidney (µg/kg)	Liver (µg/kg)	Muscle (µg/kg)
Cattle	100	400	850	250
Sheep	100	200	300	200

Tylosin (antimicrobial agent)

Acceptable daily intake: The Committee established an ADI of 0–30 µg/kg bw based on a microbiological end-point derived from in vitro MIC susceptibility testing and faecal binding data (MIC_{calc} = 1.698).

Residue definition: Tylosin A

Recommended maximum residue limits (MRLs)

Species	Fat (µg/kg)	Kidney (µg/kg)	Liver (µg/kg)	Muscle (µg/kg)	Skin/fat (µg/kg)	Milk (µg/kg)	Eggs (µg/kg)
Cattle	100	100	100	100		100	
Pigs	100	100	100	100			
Chickens		100	100	100	100		300

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 conduced 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) and 70th (2008) 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 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 70th meeting of the Committee.

Substance	ADI (µg/kg bw)	ADI Status	JECFA[1]	MRL (µg/kg)	Tissue	Species	Marker residue and other remarks
Abamectin	0-1 (JMPR 1995)	Full	47 (1996)	100 50	Liver, Fat Kidney	Cattle	Avermectin B_{1a}
Albendazole	0-50	Full	34 (1989)	100 5000	Muscle, Fat, Milk Liver, Kidney	Cattle, Sheep	MRLs analyzed as 2-amino-benzimidazole, expressed as albendazole equivalents
Avilamycin	0-2000 (as avilamycin activity)	Full	70 (2008)	200 300	Muscle, Kidney, Skin/Fat Liver	Pig, Chicken, Turkey, Rabbit Pig, Chicken, Turkey, Rabbit	Dichloroisoeverninic acid (DIA), expressed as avilamycin equivalents
Azaperone	0-6	Full	50 (1998)	60 100	Muscle, Fat Liver, Kidney	Pig	Sum of azaperone and azaperol
Benzylpenicillin	<30µg/person/ day of the penicillin moiety	Full	36 (1990)	50 4	Muscle, Liver, Kidney Milk	All species	Benzylpenicillin
Bovine Somatotropins	Not specified	Full	50 (1998)	Not specified	Muscle, Liver, Kidney, Fat, Milk	Cattle	
Carazolol	0-0.1	Full	43 (1994)	5 25	Muscle, Fat/Skin Liver, Kidney	Pig	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		60 (2003)	No MRL			The Committee decided that quinoxaline-2-carboxylic acid is not an appropriate marker residue
Ceftiofur	0-50	Full	48 (1997)	1000 2000 6000 2000 100	Muscle Liver Kidney Fat Milk	Cattle, Pig Cattle	Desfuroylceftiofur
Cefuroxime	No ADI		62 (2004)	No MRL			
Chloramphenicol	No ADI		62 (2004)	No MRL			
Chlorpromazine	No ADI		38 (1991)	No MRL			

[1] Only the last meeting of the Committee where the substance was on the agenda; earlier evaluations are referred to in the respective reports of the meetings

Substance	ADI (µg/kg bw)	ADI Status	JECFA[1]	MRL (µg/kg)	Tissue	Species	Marker residue and other remarks
Chlortetracycline Oxytetracycline Tetracycline	0-30 (Group ADI)	Full	58 (2002)	200 600 1200 400 100 200 200	Muscle Liver Kidney Eggs Milk Muscle Muscle	Cattle, Pig, Sheep, Poultry Poultry Cattle, Sheep Fish Giant prawn	Parent drugs, either singly or in combination Oxytetracycline only
Clenbuterol	0-0.004	Full	47 (1996)	0.2 0.6 0.05	Muscle, Fat Liver, Kidney Milk	Cattle, Horse Cattle	Clenbuterol
Closantel	0-30	Full	40 (1992)	1000 3000 1500 5000 2000	Muscle, Liver Kidney, Fat Muscle, Liver, Kidney Fat	Cattle Sheep	Closantel
Colistin	0-7	Full	66 (2006)	150 200 50 300	Muscle, Liver, Fat Kidney Milk Eggs	Cattle, Sheep, Goat, Chicken, Turkey, Pig, Rabbit Cattle, Sheep Chicken	Residue definition is the sum of Colistin A and colistin B. The MRL includes skin + fat where appropriate (chicken, turkey, pigs).
Cyfluthrin	0-20	Full	48 (1997)	20 200 40	Muscle, Liver, Kidney Fat Milk	Cattle	Cyfluthrin
Cyhalothrin	0-5	Full	62 (2004)	20 400 20 50 30	Muscle, Kidney Fat Liver Liver Milk	Cattle, Sheep, Pig Cattle, Pig Sheep Cattle, Sheep	Cyhalothrin
Cypermethrin α-Cypermethrin	0-20 (Group ADI)	Full	62 (2004)	50 1000 100	Muscle, Liver, Kidney Fat Milk	Cattle, Sheep Cattle, Sheep	Total of cypermethrin residues (resulting from the use of cypermethrin or α-cypermethrin as veterinary drugs)

Substance	ADI (µg/kg bw)	ADI Status	JECFA[1]	MRL (µg/kg)	Tissue	Species	Marker residue and other remarks
Danofloxacin	0-20	Full	48 (1997)	200 400 100 100 50 200 100	Muscle Liver, Kidney Fat Muscle Liver Kidney Fat	Cattle, Chicken Pig	Danofloxacin For chicken fat/skin
Deltamethrin	0-10 (1982 JMPR)	Full	60 (2003)	30 50 500 30 30	Muscle Liver, Kidney Fat Milk Eggs	Cattle, Chicken, Sheep, Salmon Cattle, Sheep, Chicken Cattle Chicken	Deltamethrin
Dexamethasone	0-0.015	Full	70 (2008)	1 2 0.3	Muscle, Kidney Liver Milk	Cattle, Pig, Horse Cattle, Pig, Horse Cattle	Dexamethasone
Diclazuril	0-30	Full	50 (1998)	500 3000 2000 1000	Muscle Liver Kidney Fat	Sheep, Rabbit, Poultry	Diclazuril Poultry skin + fat
Dicyclanil	0-7	Full	60 (2003)	150 125 200	Muscle Liver, Kidney Fat	Sheep	Dicyclanil
Dihydro-streptomycin Streptomycin	0-50 (Group ADI)	Full	58 (2002)	600 1000 200	Muscle, Liver, Fat Kidney Milk	Cattle, Pig, Chicken, Sheep Cattle, Sheep	Sum of dihydrostreptomycin and streptomycin
Dimetridazole	No ADI		34 (1989)	No MRL			
Diminazene	0-100	Full	42 (1994)	500 12000 6000 150	Muscle Liver, Kidney Milk	Cattle	Diminazene
Enrofloxacin	0-2	Full	48 (1997)	No MRL			

Substance	ADI (µg/kg bw)	ADI Status	JECFA[1]	MRL (µg/kg)	Tissue	Species	Marker residue and other remarks
Eprinomectin	0-10	Full	50 (1998)	100 2000 300 250 20	Muscle Liver Kidney Fat Milk	Cattle	Eprinomectin B_{1a}
Erythromycin	0-0.7	Full	66 (2006)	100 50	Muscle, Liver, Kidney, Fat/Skin Eggs	Chicken, Turkey Chicken	Erythromycin A
Estradiol-17β	0-0.05	Full	52 (1999)	Not specified	Muscle, Liver, Kidney, Fat	Cattle	
Febantel Fenbendazole Oxfendazole	0-7 (group ADI)	Full	50 (1998)	100 500 100	Muscle, Kidney, Fat Liver Milk	Cattle, Goat, Horses, Pig, Sheep Cattle, Sheep	Sum of febantel, fenbendazole and oxfenbendazole, expressed as oxfendazole sulfone equivalents
Fenbendazole (see Febantel)							
Fluazuron	0-40	Full	48 (1997)	200 500 7000	Muscle Liver, Kidney Fat	Cattle	Fluazuron
Flubendazole	0-12	Full	40 (1992)	10 200 500 400	Muscle, Liver Muscle Liver Eggs	Pig Poultry	Flubendazole
Flumequine	0-30	Full	66 (2006)	500 1000 500 3000 500 500T 500T	Muscle Fat Liver Kidney Muscle Muscle Muscle	Cattle, Sheep, Pig, Chicken Trout Black Tiger Shrimp Shrimp	Flumequine. The MRLs are temporary for Black Tiger Shrimp and Shrimp. The MRLs for shrimp applies to all fresh water and marine shrimp.
Furazolidone	No ADI		40 (1992)	No MRL			
Gentamicin	0-20	Full	50 (1998)	100 2000 5000 200	Muscle, Fat Liver Kidney Milk	Cattle, Pig Cattle	Gentamicin

Substance	ADI (µg/kg bw)	ADI Status	JECFA[1]	MRL (µg/kg)	Tissue	Species	Marker residue and other remarks
Imidocarb	0-10	Full	60 (2003)	300 1500 2000 50	Muscle Liver Kidney Fat, Milk	Cattle	Imidocarb, free base
Ipronidazole	No ADI		34 (1989)	No MRL			
Isometamidium	0-100	Full	40 (1992)	100 500 1000	Muscle, Fat, Milk Liver Kidney	Cattle	Isometamidium
Ivermectin	0-1	Full	58 (2002)	100 40 15 20 10	Liver Fat Liver Fat Milk	Cattle Cattle Pig, Sheep Pig, Sheep Cattle	Ivermectin B_{1a}
Levamisole	0-6	Full	42 (1994)	10 100	Muscle, Kidney, Fat Liver	Cattle, Sheep, Pig, Poultry Cattle, Sheep, Pig, Poultry	Levamisole
Lincomycin	0-30	Full	58 (2002)	200 500 1500 500 100 150	Muscle Liver Kidney Kidney Fat Milk	Chicken, Pig Chicken, Pig Pig Chicken Chicken, Pig Cattle	Lincomycin A separate MRL of 300 µg/kg for skin with 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.
Melengestrol Acetate	0-0.03	Full	66 (2006)	1 10 2 18	Muscle Liver Kidney Fat	Cattle	Melengestrol acetate
Metronidazole	No ADI		34 (1989)	No MRL			
Monensin	0-10	Full	70 (2008)	10 10 20 100 2	Muscle, Liver, Kidney Muscle, Kidney Liver Fat Milk	Chicken, Turkey, Quail Cattle, Sheep, Goat Cattle, Sheep, Goat Cattle, Sheep, Goat, Chicken, Turkey, Quail Cattle	Monensin

Substance	ADI (µg/kg bw)	ADI Status	JECFA[1]	MRL (µg/kg)	Tissue	Species	Marker residue and other remarks
Moxidectin	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	0-5	Full	70 (2008)	15 50 15T 50T	Muscle, Kidney Liver, Fat Muscle, Kidney Liver, Fat	Chicken, Pig Chicken, Pig Cattle Cattle	Narasin A Temporary MRLs for cattle, until end 2010
Neomycin	0-60	Full	60 (2003)	500 10000 500 1500	Muscle, Fat, Liver Kidney Eggs Milk	Cattle, Chicken, Sheep, Turkey Goat, Pig, Duck Cattle, Chicken, Sheep, Turkey Goat, Pig, Duck Chicken Cattle	Neomycin
Nicarbazin	0-400	Full	50 (1998)	200	Muscle, Liver, Kidney, Fat/Skin	Chicken (broilers)	N,N'-bis(4-nitrophenyl)urea
Nitrofurazone/ Nitrofural	No ADI		40 (1992)	No MRL			
Olaquindox	No ADI		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.
Oxfendazole (See Febantel)							
Oxolinic acid	No ADI		43 (1994)	No MRL			
Oxytetracycline (See chlortetracycline)							
Permethrin	No ADI		54 (2000)	No MRL			
Phoxim	0-4	Full	62 (2004)	50 400	Muscle, Liver, Kidney Fat	Goat, Pig, Sheep	Phoxim

Substance	ADI (µg/kg bw)	ADI Status	JECFA[1]	MRL (µg/kg)	Tissue	Species	Marker residue and other remarks
Pirlimycin	0-8	Full	62 (2004)	100 1000 400 100	Muscle, Fat Liver Kidney Milk	Cattle	Pirlimycin
Porcine Somatotropin	Not Specified		52 (1999)	Not Specified	Muscle, Liver, Kidney, Fat	Pig	
Procaine benzylpenicillin	< 30µg/person/ day of the penicillin moiety	Full	50 (1998)	50 4	Muscle, Liver, Kidney Milk	All species	Benzylpenicillin
Progesterone	0-30	Full	52 (1999)	Not Specified	Muscle, Liver, Kidney, Fat	Cattle	
Propionyl-promazine	No ADI		38 (1991)	No MRL			
Ractopamine	0-1	Full	66 (2006)	10 40 90	Muscle, Fat Liver Kidney	Cattle, Pig	Ractopamine
Ronidazole	No ADI		42 (1994)	No MRL			
Sarafloxacin	0-0.3	Full	50 (1998)	10 80 20	Muscle Liver, Kidney Fat/skin	Chicken, Turkey	Sarafloxacin
Spectinomycin	0-40	Full	50 (1998)	500 2000 5000 2000 200	Muscle Liver, Fat Kidney Eggs Milk	Cattle, Chicken, Pig, Sheep Chicken Cattle	Spectinomycin
Spiramycin	0-50	Full	48 (1997)	200 600 300 800 300 200	Muscle Liver Kidney Kidney Fat Milk	Cattle, Chicken, Pig Cattle, Chicken Pig Cattle, Chicken, Pig Cattle	For cattle and chicken, MRLs are expressed as the sum of spiramycin and neospiramycin. For pigs, the MRLs are expressed as spiramycin equivalents (antimicrobial active residues).
Streptomycin (See dihydro-streptomycin)							

Substance	ADI (µg/kg bw)	ADI Status	JECFA[1]	MRL (µg/kg)	Tissue	Species	Marker residue and other remarks
Sulfadimidine (Sulfamethazine)	0-50	Full	42 (1994)	100 25	Muscle, Liver, Kidney, Fat Milk	Cattle, Sheep, Pig, Poultry Cattle	Sulfadimidine
Sulfathiazole	No ADI		34 (1989)	No MRL			
Testosterone	0-2	Full	52 (1999)	Not specified	Muscle, Liver, Kidney, Fat	Cattle	
Tetracycline (See chlortetracycline)							
Thiamphenicol	0-5	Full	58 (2002)	No MRL			
Tiabendazole (Thiabendazole)	0-100	Full	58 (2002)	100 100	Muscle, Liver, Kidney, Fat	Cattle, Pig, Goat, Sheep Cattle, Goat	Sum of tiabendazole + 5-hydroxy tiabendazole
Tilmicosin	0-40	Full	70 (2008)	100 1000 1500 300 1000 150 100 2400 1400 600 1200 250	Muscle, Fat Liver Liver Kidney Kidney Muscle Muscle Liver Liver Kidney Kidney Skin/Fat	Cattle, Pig, Sheep Cattle Sheep Pig Cattle, Sheep Pig Chicken Turkey Chicken Turkey Chicken Turkey Chicken, Turkey	Tilmicosin
Trenbolone acetate	0-0.02	Full	34 (1989)	2 10	Muscle Liver	Cattle	β Trenbolone for muscle α-Trenbolone for liver
Trichlorfon (Metrifonate)	0-2	Full	66(2006)	50 50	Milk Muscle, Liver, Kidney, Fat	Cattle	Trichlorfon 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.

Substance	ADI (µg/kg bw)	ADI Status	JECFA[1]	MRL (µg/kg)	Tissue	Species	Marker residue and other remarks
Triclabendazole	0-3	Full	70 (2008)	250 850 400 200 300 200 100	Muscle Liver Kidney Muscle Liver Kidney Fat	Cattle Sheep Cattle, Sheep	Keto-triclabendazole
Tylosin	0-30	Full	70 (2008)	100 100 100 100 300	Muscle, Liver, Kidney Fat Skin/Fat Milk Eggs	Cattle, Pig, Chicken Cattle, Pig Chicken Cattle Chicken	Tylosin A
Xylazine	No ADI		47 (996)	No MRL			
Zeranol	0-0.5	Full	32 (1987)	2 10	Muscle Liver	Cattle	Zeranol

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RESIDUE EVALUATION OF CERTAIN VETERINARY DRUGS

Joint FAO/WHO Expert Committee on Food Additives

70th meeting 2008

This document contains monographs on residue evaluations of certain veterinary drugs, prepared at the seventieh meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), which was held in Geneva, Switzerland from 21 to 29 October 2008. Three substances were evaluated for the first time for the animal species concerned, avilamycin, monensin and narasin. Four substances were reassessed, dexamethasone, tilmicosin, triclabendazole and tylosin. The residue monographs provide 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. In addition to these monographs, this document includes the considerations and recommendations developed by the Committee for residues of veterinary drugs in honey and possible approaches to derive MRLs for this commodity; and a monograph on residues in aquatic species of and an estimatation of human dietary exposure to malachite green (an antimicrobial agent and contamimant). This publication and other documents produced by JECFA contain information that is useful to those who work with or are involved with recommending or controlling maximum residue limits for veterinary drugs in foods.

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