NARASIN

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IDENTITY

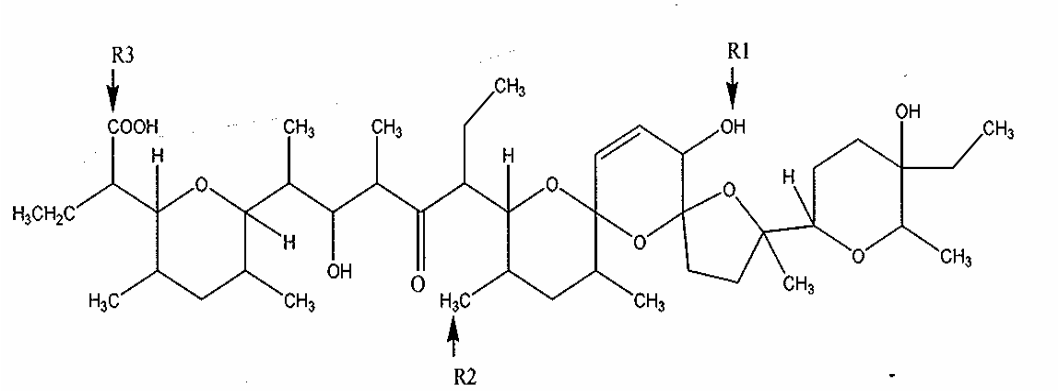
International Non-proprietary names (INN): Narasin

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.

REFERENCES

Catherman, D.R., Szabo, J., Batson, D.B., Cantor A.H., Tucker, R.E., and Mitchell, G.E. (1991). Metabolism of Narasin in Chickens and Japanese Quail. Poultry Science, 70, 120-125.

Donoho, A.L., Herberg, M.S., and Thomson, T.D. (1988). ^{14}C Narasin tissue residue study in pigs. Agricultural Biochemistry. Lilly Research Laboratories, Division of Eli Lilly and company, Report Number ABC-0392. Sponsor submitted.

Dubois, M., Pierret, G., and Delahaut, P. (2004). Efficient and sensitive detection of residues of nine coccidiostats in egg and muscle by liquid chromatography-electrospray tandem mass spectrometry. Journal of Chromatography *B,* 813, 181-189.

Handy, P.R., Thomson, T.D., and Tamura, R.N. (1985). Determination of the depletion of narasin residues in broiler chickens. Agricultural Analytical Chemistry, Lilly Research Laboratories, Division of Eli Lilly and Company, Report Number AAC-8408. Sponsor submitted.

Holmstrom, S.D., Kiehl, D.E., Fossler, S.C., and Clark, K.J. (2002). ^{14}C Narasin residue and metabolism in broiler chickens. Animal Health Chemistry Research. Lilly Research Laboratories, Division of Eli Lilly and Company, Report Number T4H610101. Sponsor submitted.

Lacoste, E., and Larvor, A. (2003). Residue study in edible tissues of broiler chickens fed with narasin at 80 ppm for five consecutive days. European Animal Science Research. Elanco Animal Health, Division of Eli Lilly and Company, Report Number T2NAFR0103. Sponsor submitted.

Manthey, J.A. (1977a). Excretion of ^{14}C narasin by chickens and rats. Agricultural Biochemistry. Lilly Research Laboratories, Division of Eli Lilly and Company, Report Number Q61-3414 & Q61-3422-68. Sponsor submitted.

Manthey, J.A. (1977b). Tissue residue and residue depletion studies with ^{14}C Narasin in chickens. Agricultural Biochemistry. Lilly Research Laboratories, Division of Eli Lilly and Company. Sponsor submitted.

Manthey, J.A., Herberg, R.J., Handy, P.R., and Van Duyn, R.L. (1981). Determination of levels of tissue residues and the rate of decline of residues from tissues of chickens dosed orally for five days with 100 ppm ^{14}C Narasin ration. Agricultural Biochemistry. Lilly Research Laboratories, Division of Eli Lilly and Company, Report Number ABC-0093, Sponsor submitted.

Manthey, J.A., and Goebel, G.V. (1982). Isolation and characterization of narasin metabolites derived from excreta of orally dosed chickens. Agricultural Biochemistry, Lilly Research Laboratories, Division of Eli Lilly and Company. Sponsor submitted.

Manthey, J.A., Herberg, R.J., and Van Duyn, R.L. (1982). A ^{14}C Narasin tissue residue and comparative metabolism study in cattle. Agricultural Biochemistry. Lilly Research Laboratories, Division of Eli Lilly and Company, Report Number ABC-0137. Sponsor submitted.

Manthey, J.A., Herberg, R.J., Mattingly, C.L., Hanasono, G.K., and Donoho, A.L. (1983). ^{14}C Narasin tissue residue bioavailability study. Agricultural Biochemistry. Lilly Research Laboratories, Division of Eli Lilly and Company, Report Number ABC-0150. Sponsor submitted.

Manthey, J.A., Herberg, R.J., and Van Duyn, B.S. (1984a). ^{14}C Narasin excretion study in cattle. Agricultural Biochemistry. Lilly Research Laboratories, Division of Eli Lilly and Company, Report Number ABC-0125. Sponsor submitted.

Manthey, J.A., Herberg, M.S., and Thomson, T.D. (1984b). A study to determinate the rate of decline of ^{14}C residues from edible tissues of cattle dosed orally for five days with ^{14}C Narasin. Agricultural Biochemistry. Lilly Research Laboratories, Division of Eli Lilly and Company, Report Number ABC-0264. Sponsor submitted.

Manthey, J.A., and Goebel, G.V. (1986). Comparative metabolism of ^{14}C Narasin in orally dosed cattle, dog and rats. Agricultural Biochemistry. Lilly Research Laboratories, Division of Eli Lilly and company, Report Number ABC-0126, ABC-0127.Sponsor submitted.

Maruyama, N., and Sugimoto, T. (2000). Narasin residue trial in broiler chicken-I. Research Institute for Animal Science in Biochemistry and Toxicology, Lilly Japan K.K, Division of Eli Lilly and company, Report Number T2NJA9837. Sponsor submitted.

Matabudul, D., Lumley, I.D. and Points, J. (2002). The determination of 5 anticoccidial drugs (nicarbazin, lasolacid, monensin, salinomycin, and narasin) in animal livers and eggs by liquid chromatography linked with tandem mass spectrometry (LC-MS-MS). The Analyst, 127, 760-768.

Moran, J.M., Donoho, A.L., and Coleman, M.R. (1992). Narasin tissue residue study in growing-finishing pigs. Animal Science Chemical Research. Lilly Research Laboratories, Division of Eli Lilly and company, Report Number T6KCA9201. Sponsor submitted.

OIE (World Organisation for Animal Health) (2007). OIE List of antimicrobials of veterinary importance. Adopted by the 75th General Session of OIE, May 2007 (Resolution No. XXVIII). Available at the website of OIE at:
http://www.oie.int/downld/Antimicrobials/OIE_list_antimicrobials.pdf (Accessed 9 February 2009).

Peippo, P., Hagren, V., Lovgren, T., and Tuomola, M. (2004). Rapid time-resolved fluoroimmunoassay for the screening of narasin and salinomycin residues in poultry and eggs. J. Agric. Food Chem., 52, 1824-1828.

Peippo, P., Lovgren, T., and Tuomola, M. (2005). Rapid screening of narasin residues in poultry plasma by time-resolved fluoroimmunoassay. Analytica Chimica Acta, 529, 27–31.

Potter, E.L., and Cooley C.O. (1975). Study of the residues of orally administered Narasin in the tissue of fattening cattle. Agricultural Analytical Chemistry. Lilly Research Laboratories. Division of Eli Lilly and Company. Report Number CA-264. Sponsor submitted.

Rokka, M., and Peltonen, K. (2006). Simultaneous determination of four coccidiostats in eggs and broiler meat: Validation of an LC-MS/MS method. Food Additives and Contaminants, 23, 470-478.

Sweeney, D.J., and Kennington, A.S. (1994). Narasin metabolite study with rat faeces. Animal Science Chemical Research, Lilly Research Laboratories, Division of Eli Lilly and Company, Report Number TAH969401. Sponsor submitted.

Sweeney, D.J., Kennington, A.S., and Darby, J.M. (1994). A comparative metabolism study in tissues and excreta of chickens dosed with ^{14}C Narasin with and without Nicarbazin. Animal Science Chemical Research. Lilly Research Laboratories, Division of Eli Lilly and company, Report Number T4H969301. Sponsor submitted.

Sweeney, D.J., Kennington, A.S., Buck, J.M., Ehrenfried, K.M., and Kiehl, D.E. (1995). ^{14}C Narasin tissue residue and metabolism study in swine. Animal Science Product Development. Lilly Research Laboratories, Division of Eli Lilly and Company, Report Number T6M969501. Sponsor submitted.

Ward, T.L., Moran, J.W., Turner, J.M., and Coleman, M.R. (2005). Validation of a method for the determination of narasin in the edible tissues of chickens by liquid chromatography. J. AOAC Int., 88: 95-101.

WHO (World Health Organization) (2007). Critically important antibacterial agents for human medicine: caterogirization for the development of risk management strategies to contain antimicrobial resistance due to non-human antimicrobial use. Report of the second WHO expert meeting, Copenhagen, Denmark, 29-31 May, 2007. Available at the website of WHO at: http://www.who.int/foodborne_disease/resistance/antimicrobials_human.pdf (Accessed 9 February 2009).

TILMICOSIN

First draft prepared by
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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

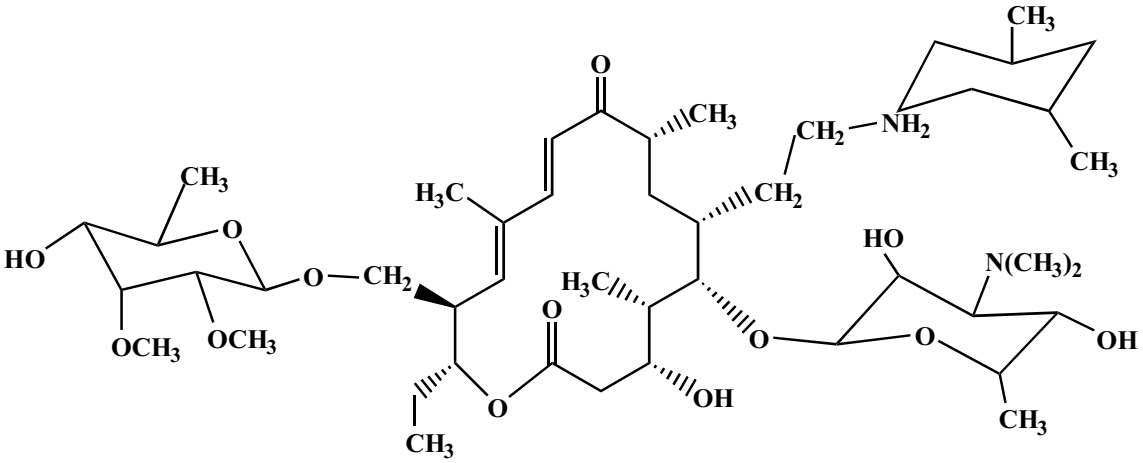
CAS Name: Tylosin,A-O-de(2,6-dideoxy-3-C-methyl-alpha-L-ribo-hexopyranosyl)-20-deoxy-20-(3,5-dimethyl-1-piperidinyl)-(20(cis: trans))

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

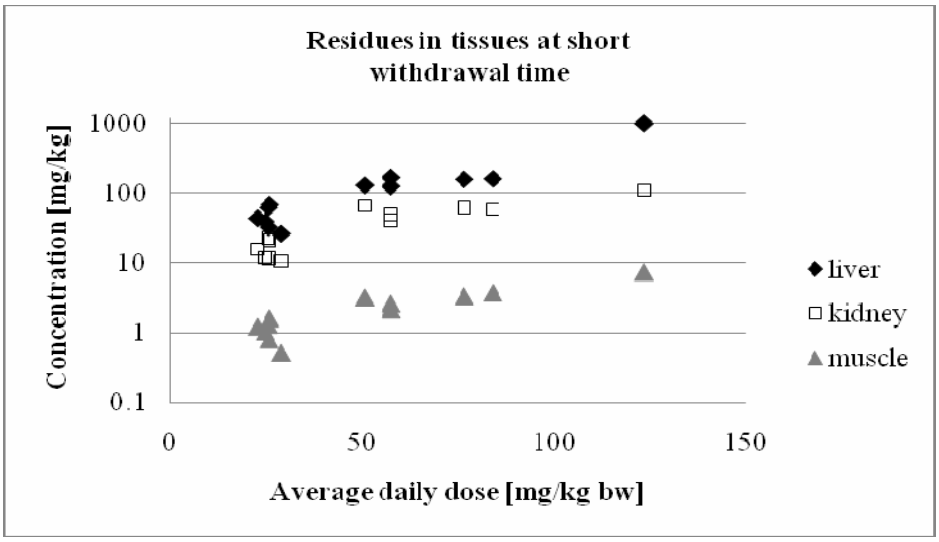
Table 4: Results of the tissue analyses carried out in study T5C749504.

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.

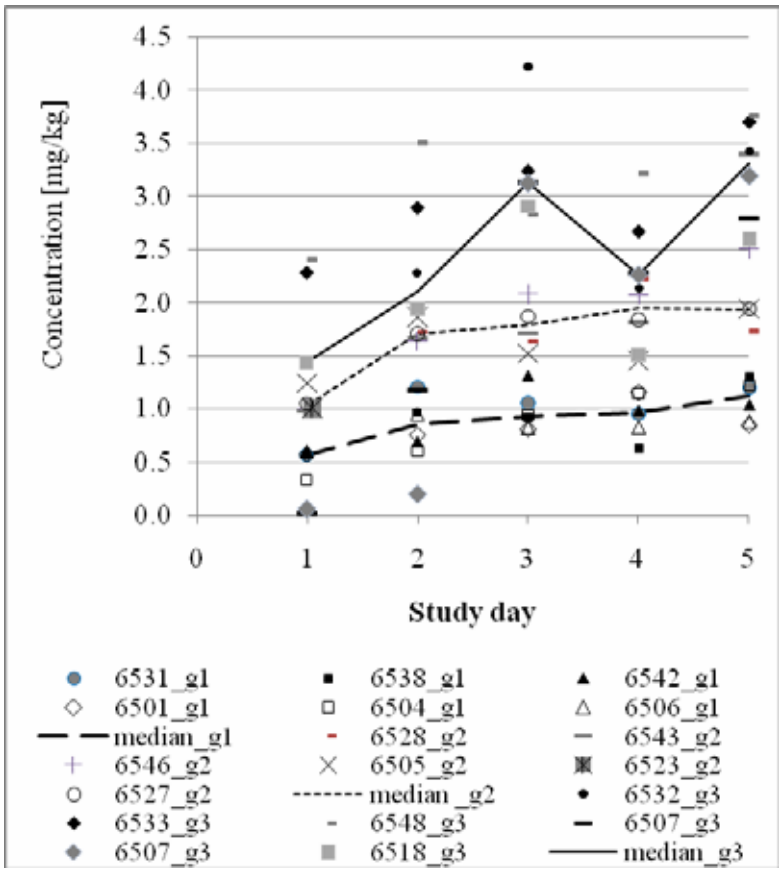
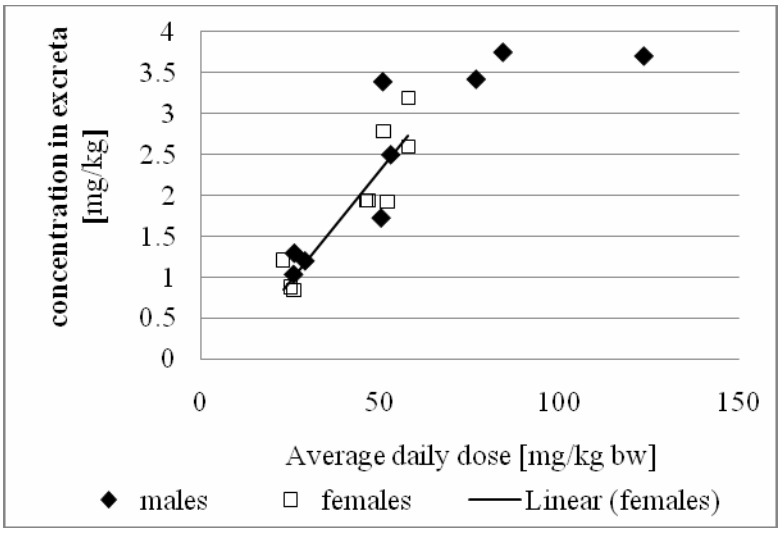


Figure 3: Day 5 concentration of radioactive residues [tilmicosin equivalents] in excreta.



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						

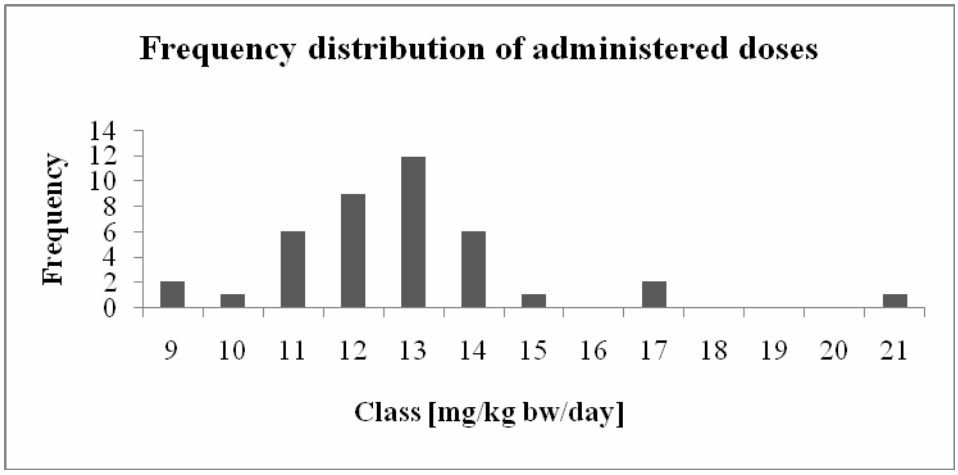
In another GLP-compliant study with ^{14}C-tilmicosin in chicken (T5C749602; Ehrenfried, et al, 1997b), five groups of eight (4 of each sex) 4-week old Cornish Cross chickens received [^{14}C]-tilmicosin (specific activity 2.62 µCi/mg) in medicated drinking water at concentration of 75 mg/L ad libitum 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.



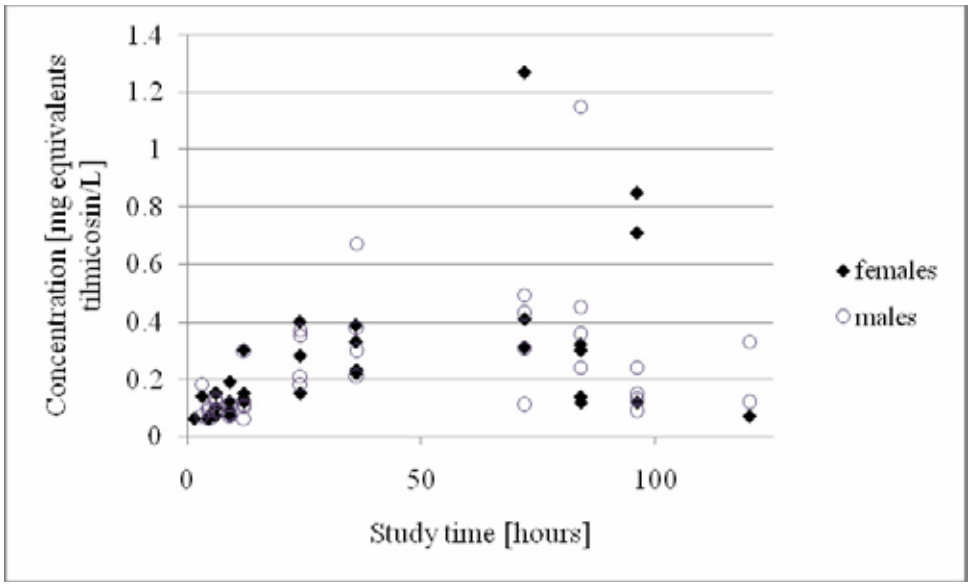
Another GLP compliant study (96 – ELA – 01, Peters, et al., 1997) involved 184 broiler chickens (92 of each sex). At an age of 3 – 4 weeks groups of animals were treated using three concentrations of tilmicosin in drinking water. Water was provided *ad libitum* from hanging drinkers. Body weight ranged 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

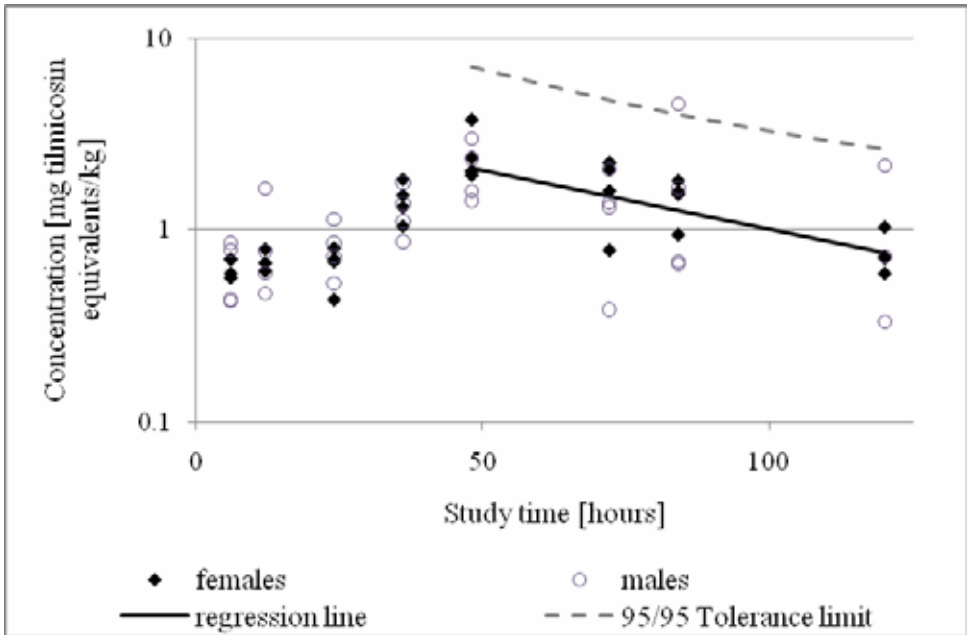
Serial blood samples were taken from the wing veins of eight birds of each group from time 0 to 120 hours after treatment. If both wing veins collapsed of developed hematomas spare chickens were used. Some haemolysed samples could not be used for the analyses. In addition, samples with a volume below 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

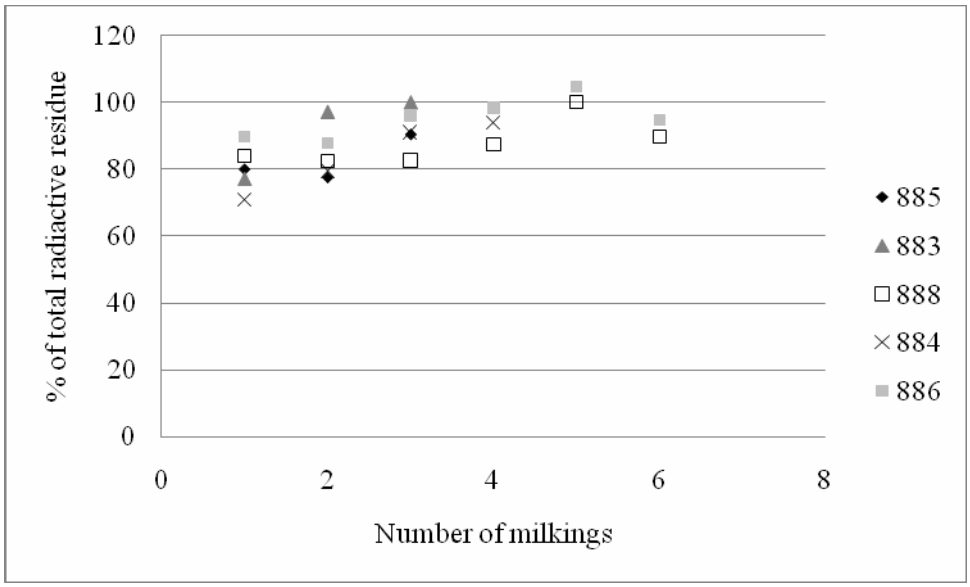
Studies in milk producing animals

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.

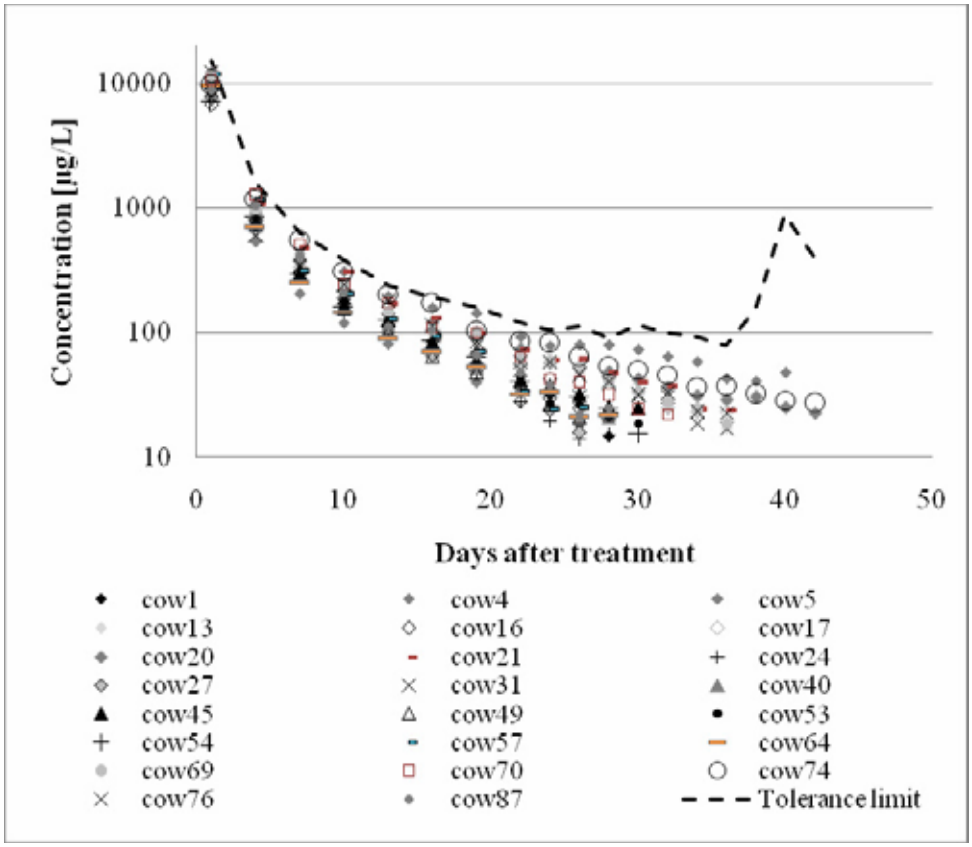


Studies with unlabelled tilmicosin

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.

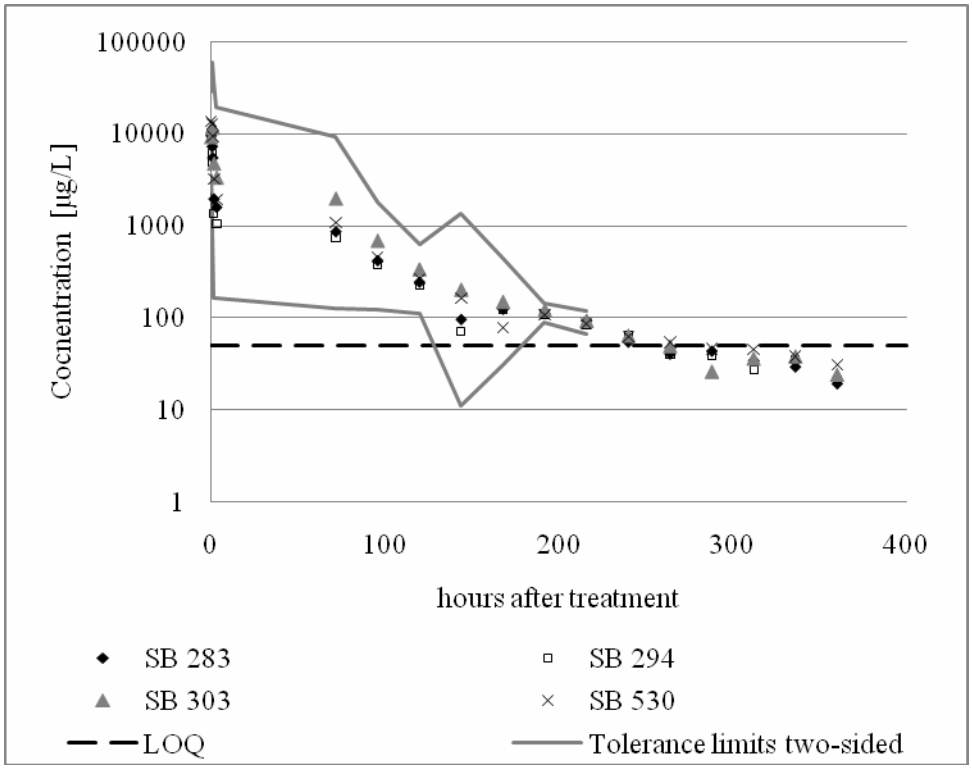


Sheep

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.

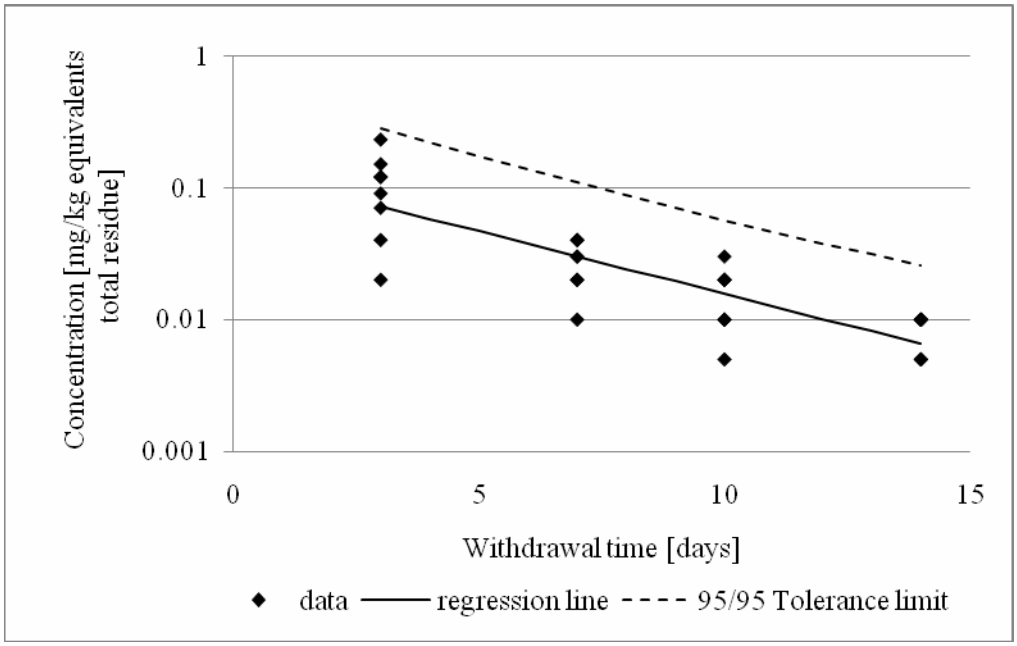
Chickens

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

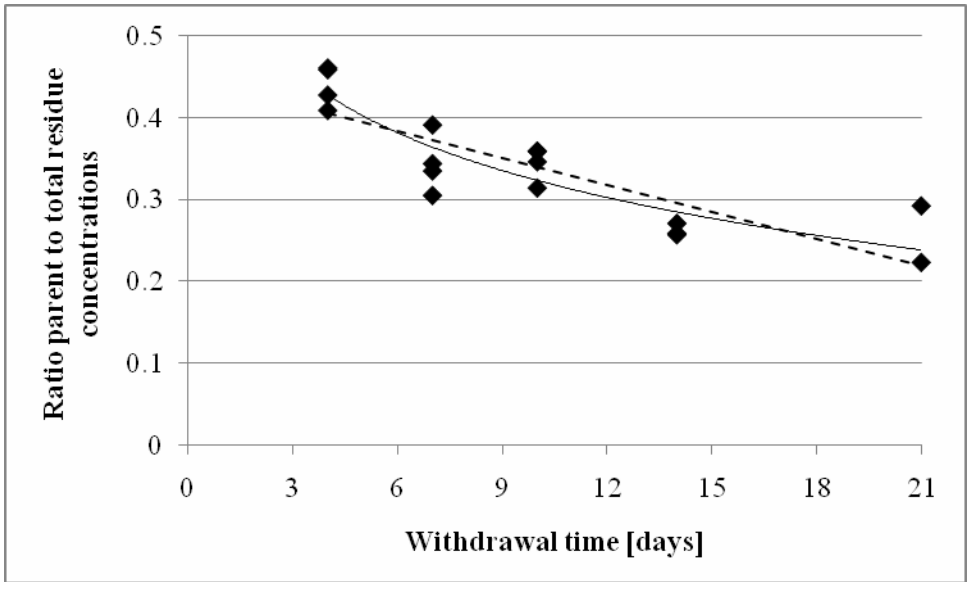
These values may need to be adjusted depending on the dose resulting from authorised treatments according to the label instructions. The information on the Irish label suggests a range of daily doses from 10 to 25 mg/kg of body weight. The French and Swiss labels assume a range of 15 to 20 mg/kg bw per 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.

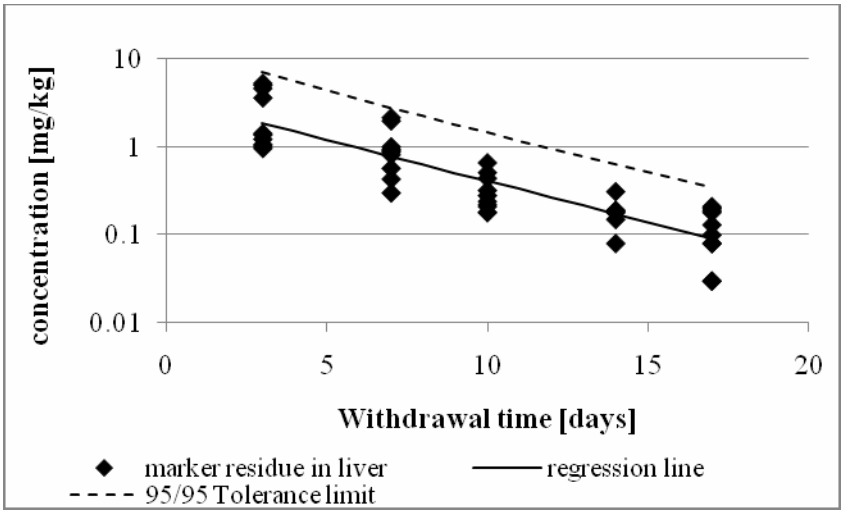
day	% parent tilmicosin in extract	
	Liver	Kidney
3	49.5	19.2
7	46.5	6.3
10	37.6	11.1
14	26.2	2.7
21	18.9	7.2

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									

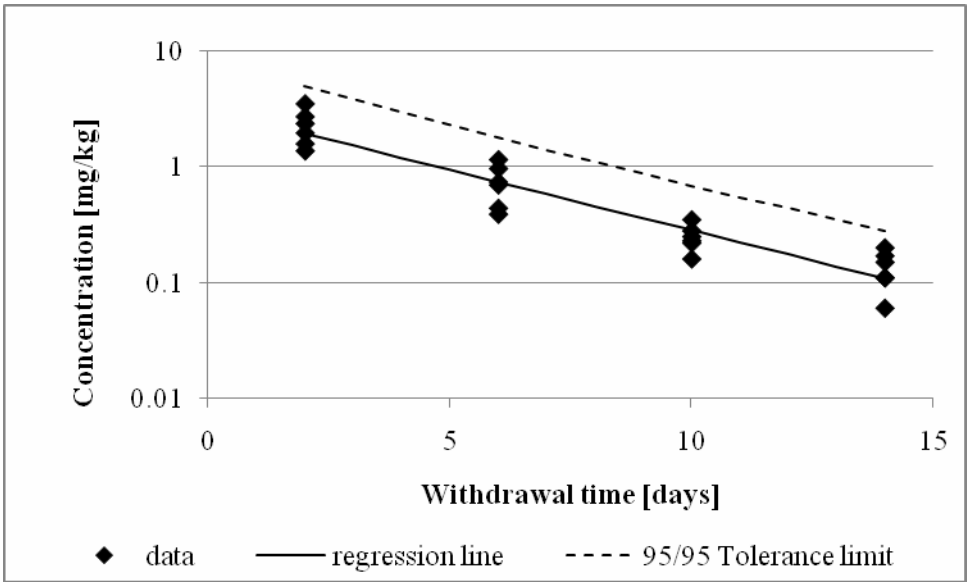
Turkey

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.

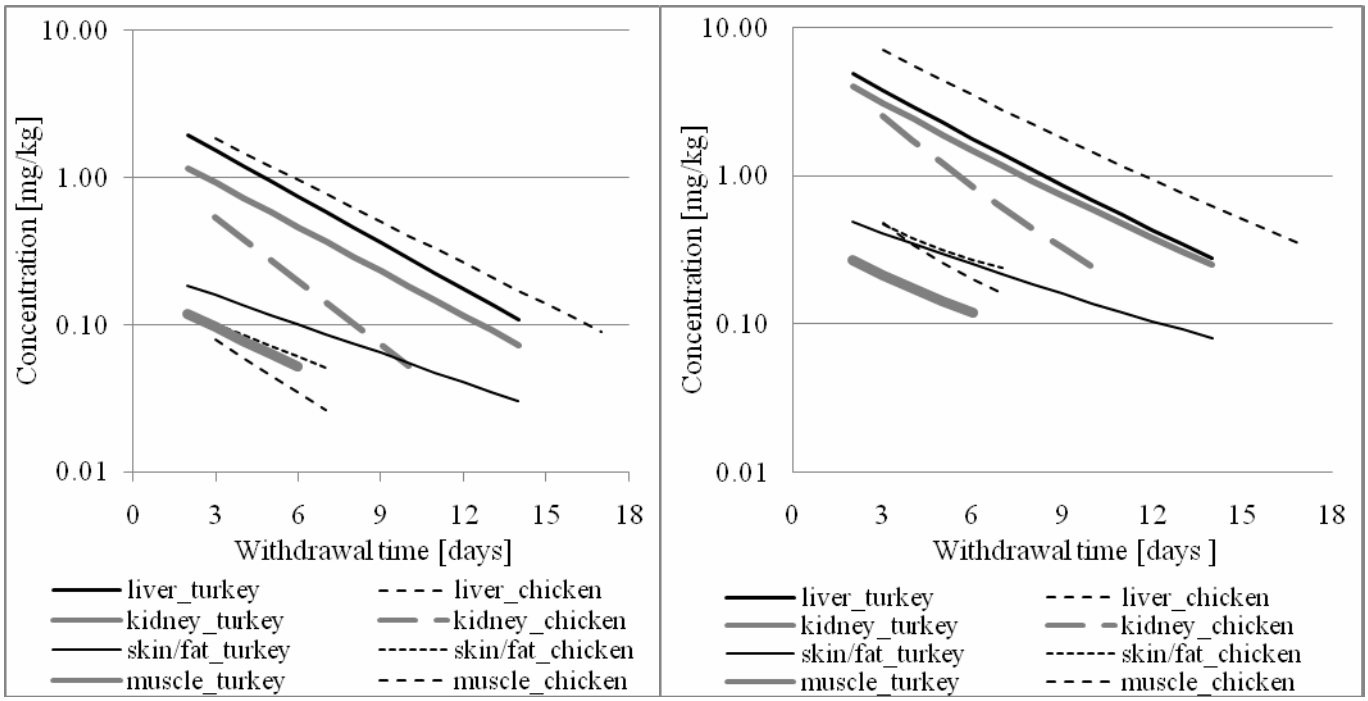


Table 18: Basis for recommending MRLs in turkeys.

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.

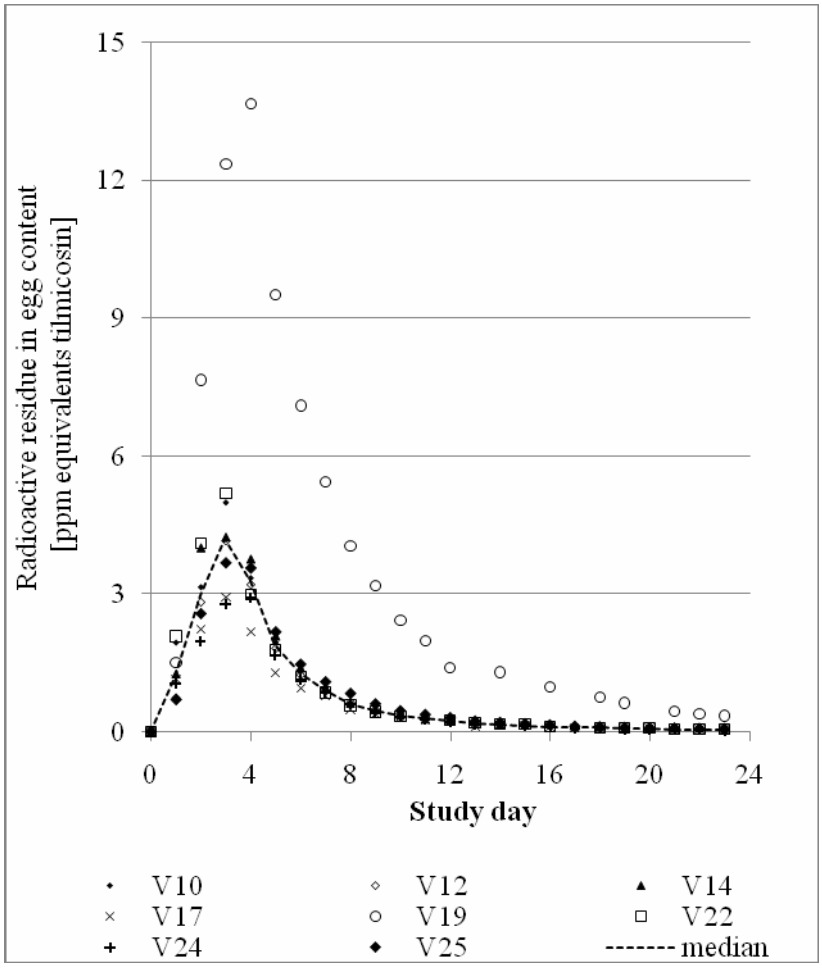


Table 20: Ratio of marker to total residue concentrations.

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.

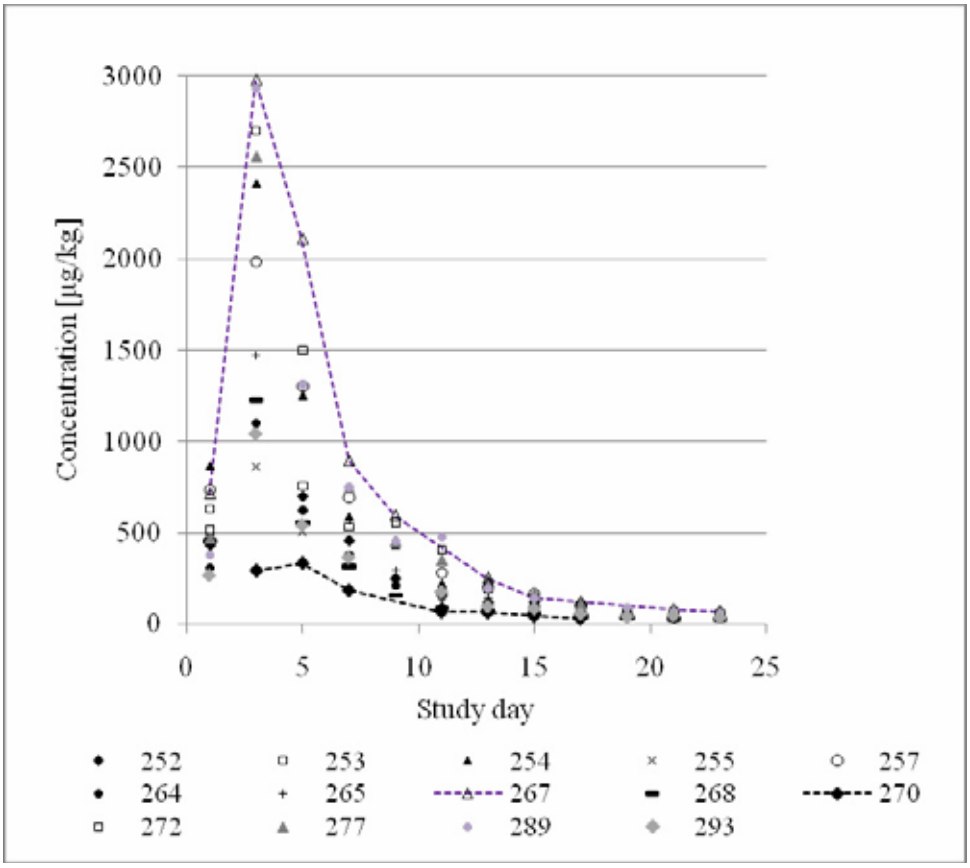


Table 22: Statistical evaluation of the laying hen eggs data.

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.

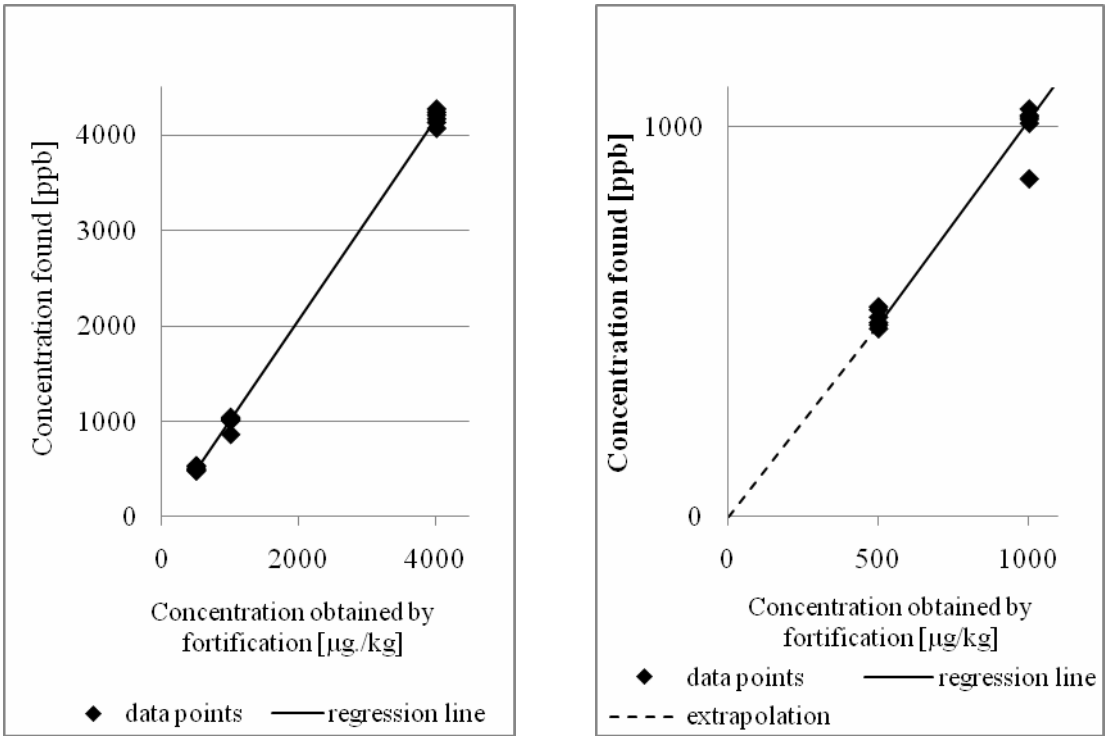
Rabbits

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.

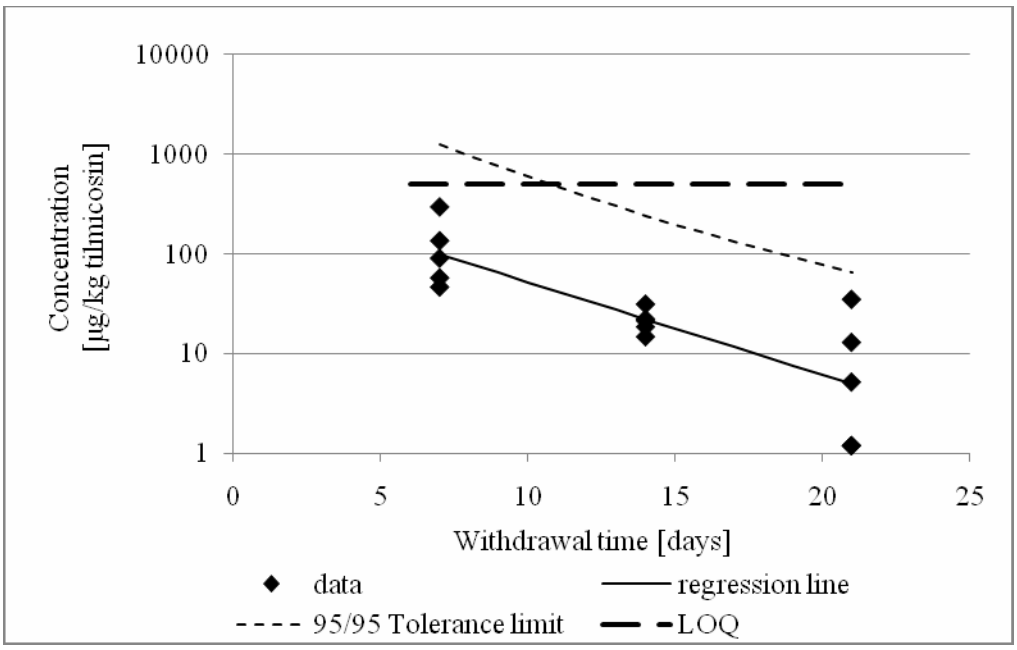
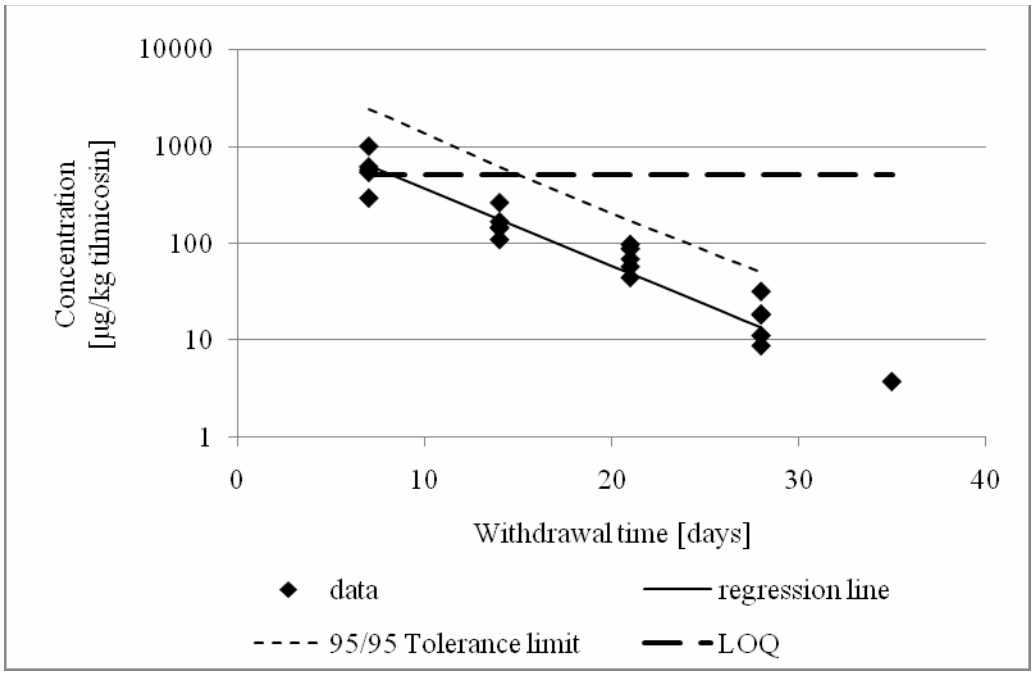


Figure 19: Relationship of the measurements in kidney of rabbits to the LOQ.



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.

REFERENCES

Beauchemin, V., Byrd, J., Burnett T., and Grant T.D. (2007a). ^{14}C-Tilmicosin residue and metabolism in chicken eggs. Eli Lilly and Company, Indianapolis, IN, USA. Sponsor submitted.

Beauchemin, V., Byrd, J., and Burnett, T. (2007b). Tilmicosin residue depletion study in chicken eggs. Eli Lilly and Company, Indianapolis, IN, USA. Sponsor submitted.

Donoho, A.L., and Thomson, T.D. (1990). [^{14}C]Tilmicosin milk residue study in dairy cows. Unpublished Study No. ABC-0447 from Elanco Animal Health, a division of Eli Lilly and Company, Greenfield, IN, USA. Sponsor submitted.

FAO/WHO (1998). Evaluation of Certain Veterinary Drug Residues in Foods (Forty-seventh Report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series No. 876.

Ehrenfried, K.M., Bridges, D.A., Fossler, S.C., and Kennington, A.S. (1996a). ^{14}C-Tilmicosin drinking water medication residue study in chickens. Study T5C749505. Unpublished study from the Animal Science Product Development, Elanco Animal Health Research and Development, a division of Eli Lilly and Company, Greenfield, IN, USA. Sponsor submitted.

Ehrenfried, K.M., Fossler, S.C., and Kennington, A.S. (1996b). ^{14}C-Tilmicosin drinking water medication residue study in chickens. Study T5C749601. Unpublished study from the Animal Science Product Development, Elanco Animal Health Research and Development, a division of Eli Lilly and Company, Greenfield, IN, USA. Sponsor submitted.

Ehrenfried, K.M., Buck, J.M., Kiehl, D.E., Grundy, J.S., Stobba-Wiley, C.M., and Kennington, A.S. (1997a). [^{14}C]Tilmicosin drinking water medication residue and metabolism study in chickens. Study T5C749504. Unpublished study from the Animal Science Product Development, Elanco Animal Health Research and Development, a division of Eli Lilly and Company, Greenfield, IN, USA. Sponsor submitted.

Ehrenfried, K.M., Kiehl, D.E., Fossler, S.C., Grundy, J.S., Stobba-Wiley, C.M., and Kennington, A.S. (1997b). [^{14}C]Tilmicosin drinking water medication residue decline and metabolism study in chickens. Study T5C749602. Unpublished study from the Animal Science

Product Development, Elanco Animal Health Research and Development, a division of Eli Lilly and Company, Greenfield, IN, USA. Sponsor submitted.

Ehrenfried, K.M., Kiehl, D.E., and Grundy, J.S. (1998). Assay of turkey livers for parent tilmicosin from trial DK9705T14. Eli Lilly Master Code 870. Unpublished study from the Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Sponsor submitted.

Hawthorne, P. (1999). Validation of an analytical method for the determination of tilmicosin residues in turkey liver, kidney, muscle and skin/fat samples, Unpublished Study Number CEMS-1035 CEM Analytical Services, Berkshire, England for Elanco Animal Science Research, Lilly Industries Limited, Basingstoke, UK. Sponsor submitted.

Lacoste, E. (2003). Residue study of tilmicosin in bovine milk following a single subcutaneous administration of Micotil® 300 in dairy cattle. Unpublished Study No. A03586/T5CCFF0301 from Avogadro Labs, Fontenilles, France conducted for Elanco Animal Health, Eli Lilly and Company, Suresnes Cedex, France. Sponsor submitted.

Lilly Method B04228 rev 7. Lilly Laboratory Procedure for Method B04228 revision 7. Determination of tilmicosin residues in chicken, swine, cattle and sheep edible tissues by high performance liquid chromatography. Sponsor submitted.

Luperi, L., and Brightwell, J. (1999a). Tilmicosin tissue residue study in the rabbit. Unpublished Study no. RTC 6483 (Eli Lilly Study No. T5CRIT9801) from RTC Research Toxicology Centre S.p.A., Roma for Eli Lilly Italia S.p.A., Sesto Fiorentino Italy. Sponsor submitted.

Luperi, L., and Brightwell, J. (1999b). Tilmicosin in rabbit tissues set up and validation of the analytical method. Unpublished RTC Study no.: 6484/T/219/98 from RTC Research Toxicology Centre S.p.A., Roma for Eli Lilly Italia S.p.A., Sesto Fiorentino Italy. Sponsor submitted.

McCracken, B. (2007). Validation of the analytical method, "Method of analysis for the determination of tilmicosin in whole chicken egg by LC/MS/MS", Study P0002796 includes Appendix 1 Analytical Method V003516, Method of analysis for the determination of tilmicosin in whole chicken eggs by LC/MS/MS from, MPI Research, Inc., State College, PA, USA. Sponsor submitted.

Montesissa, C., Capolongo, F., Santi, A., Biancotto, G., and Dacasto, M. (2004). Metabolism of tilmicosin by rabbit liver microsomes and hepatocyte. The Veterinary Journal, 167, 87-94.

Modric, S. Webb, A.I., and Derendorf, H. (1998). Pharmacokinetics and pharmacodynamics of tilmicosin in sheep and cattle. J.vet. Pharmacol. Therap., 21, 444-452.

MPI Method V0003516. Analytical Method V0003516, Method of analysis for the determination of tilmicosin in whole chicken egg by LC/MS/MS, MPI Research, Inc., State College, PA, USA. Sponsor submitted.

Parker, R.M., Walker, A.M., and McLaren, I.M. (1992). Tilmicosin: milk depletion study in sheep. Unpublished Study No.CVLS3/92 from CVL, Weybridge, UK conducted for Elanco Animal Health, Eli Lilly and Company, Basingstoke, UK. Sponsor submitted.

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.

Ramadan, A. (1997). Pharmacokinetics of tilmicosin in serum and milk goats. Research in Veterinary Science 62, 48-50

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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.

Warren, M.J. (2000). Tilmicosin residue depletion study in turkeys following oral administration via the drinking water. Unpublished Study Number TUR-99-10 & Trial Number T5DTUK0001 from the Royal Veterinary College, UK. Sponsor submitted.

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.

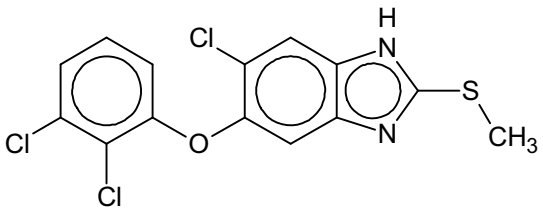
IDENTITY

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.

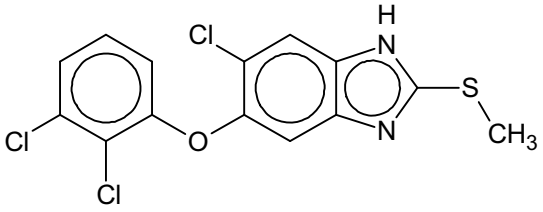
The 66th meeting defined the marker residue as "keto-triclabendazole" and recommended the following Maximum Residue Limits (µg/kg):

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.:

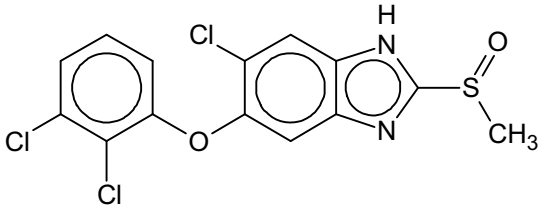
Species	MRLs in Tissues (µg/kg)			
	Muscle	Liver	Kidney	Fat
Cattle	275	600	375	200
Sheep	275	600	375	200
Goat	275	600	375	200

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:



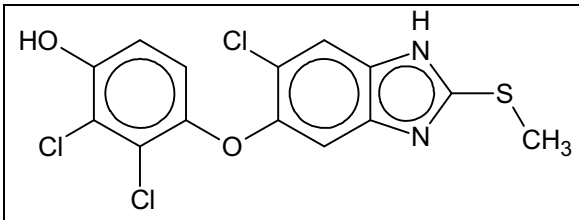
6-chloro-5-(2,3-dichlorophenoxy)-2-(methylthio)-1*H*-benzimidazole
Molecular Formula = $C_{14}H_9Cl_3N_2OS$
Formula Weight = 359.65
Synonyms and abbreviations are:
CGA 89 317 and CGP 23 030.

Triclabendazole



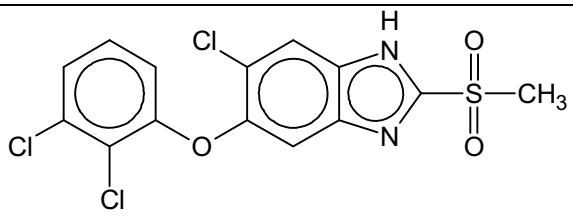
6-chloro-5-(2,3-dichlorophenoxy)-2-(methylsulfinyl)-1*H*-benzimidazole
Molecular Formula = $C_{14}H_9Cl_3N_2O_2S$
Formula Weight = 375.65
Synonyms and abbreviations are:
CGA 110 752

Triclabendazole sulphoxide



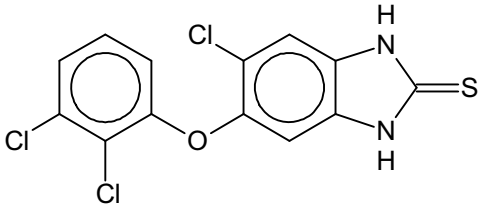
2,3-dichloro-4-{[6-chloro-2-(methylthio)-1*H*-benzimidazol-5-yl]oxy}phenol
Molecular Formula = $C_{14}H_9Cl_3N_2O_2S$
Formula Weight = 375.65
Synonyms and abbreviations are:
CGA 161 944

4-Hydroxytriclabendazole

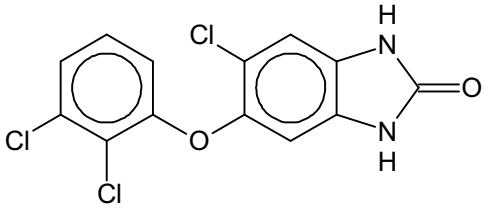


6-chloro-5-(2,3-dichlorophenoxy)-2-(methylsulfonyl)-1*H*-benzimidazole
Molecular Formula = $C_{14}H_9Cl_3N_2O_3S$
Formula Weight = 391.65
Synonyms and abbreviations are:
CGA 110 753

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

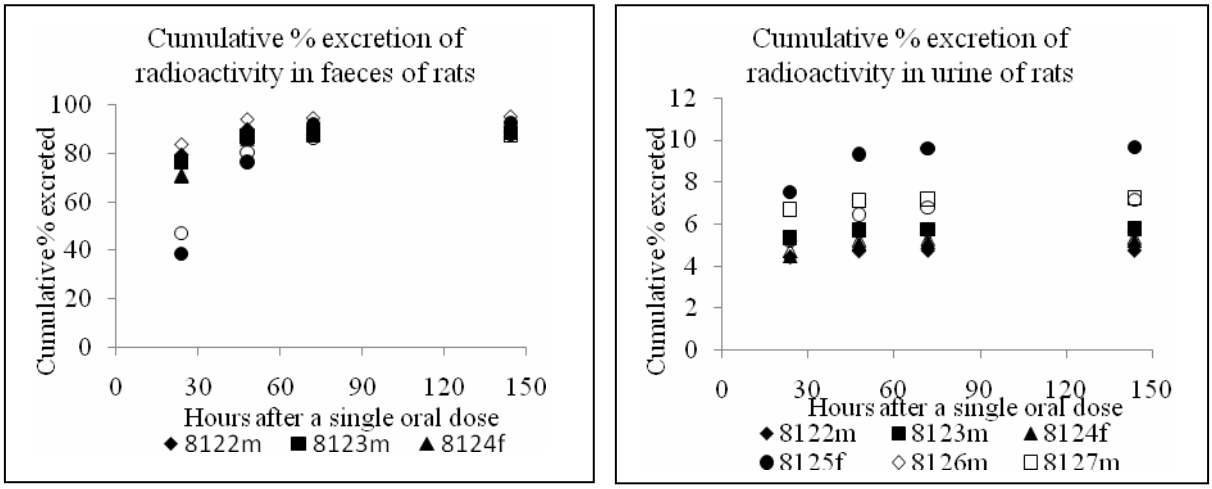
counting. Samples of faeces were extracted with methanol/water 80:20 and subjected to co-chromatography on TLC plates with reference standards.

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.

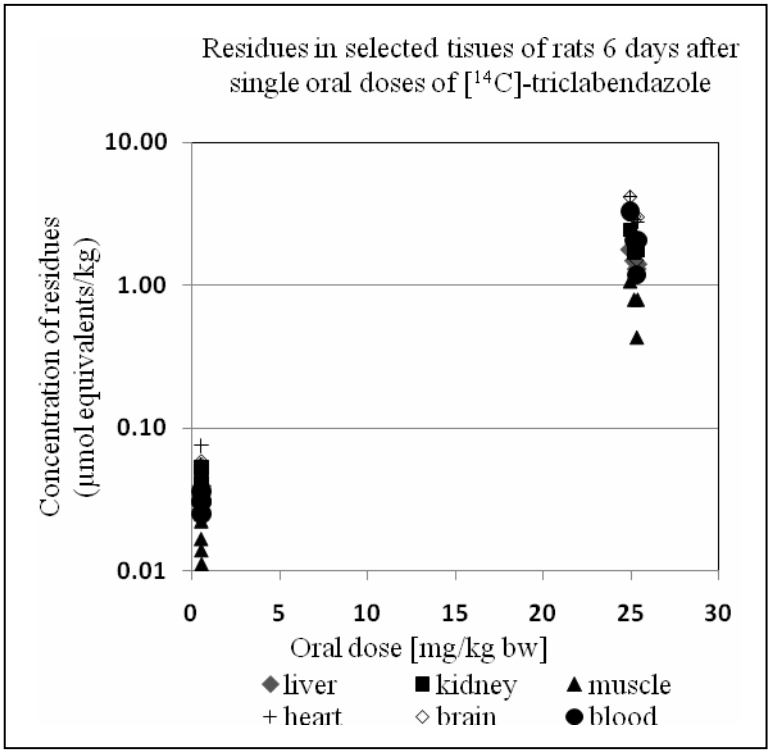


The code numbers in the legend refer to animal ID; m = male; f = female. The filled symbols indicate results obtained with the lower dose (approximately 0.5 mg/kg of bw); the open symbols indicate results obtained with the higher dose (approximately 25 mg/kg of bw).

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.

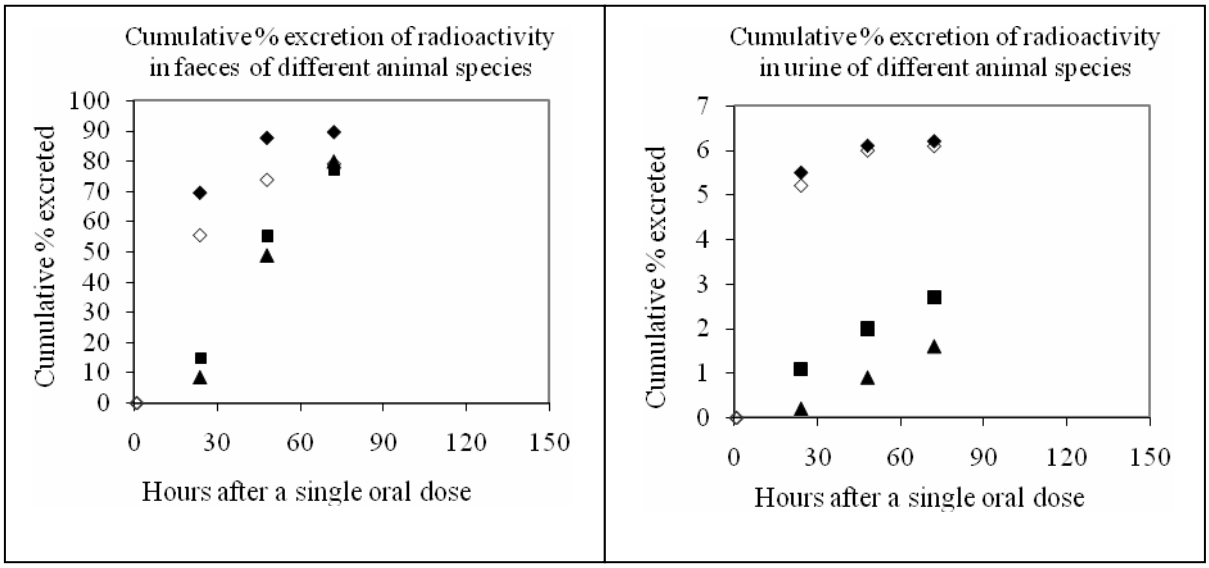
Figure 2: ^{14}C residues in tissues of rats six days after a single oral dose of approximately 25 mg [^{14}C]-triclabendazole/kg of bw.



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

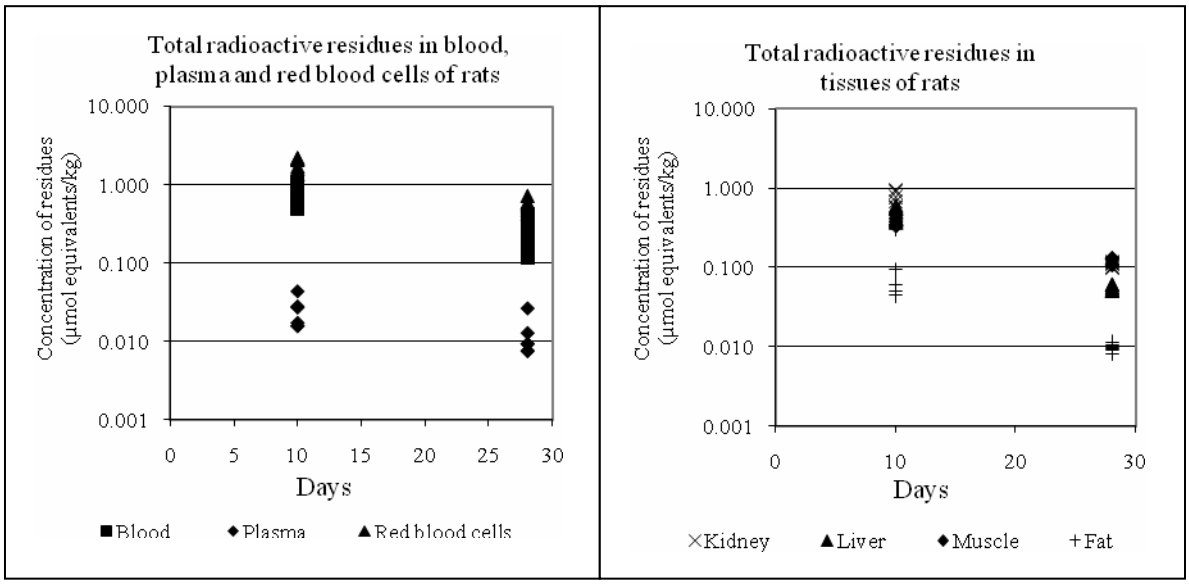
Excretion balance and tissue distribution studies (Hardwick, 2004a) were conducted in twelve Sprague Dawley rats dosed orally by gavage at a nominal dose rate of 12 mg (range 10.32-12.14 mg) triclabendazole per kg of bw. Triclabendazole was labelled in the benzene ring of the benzimidazole moiety (specific 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.



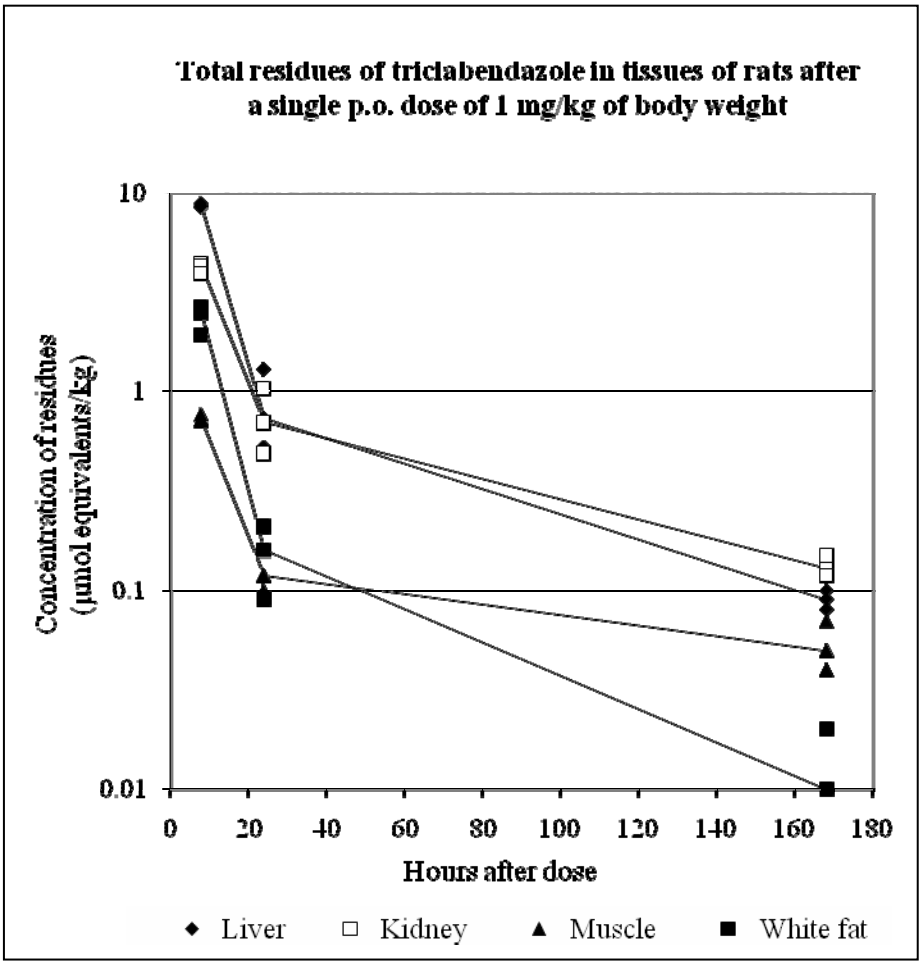
The results of studies into the extractability following NaOH hydrolysis are shown in Table 4 and indicate that 2M NaOH was equally efficient in solubilising parts of the residues in red blood cells, liver and kidney.

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).

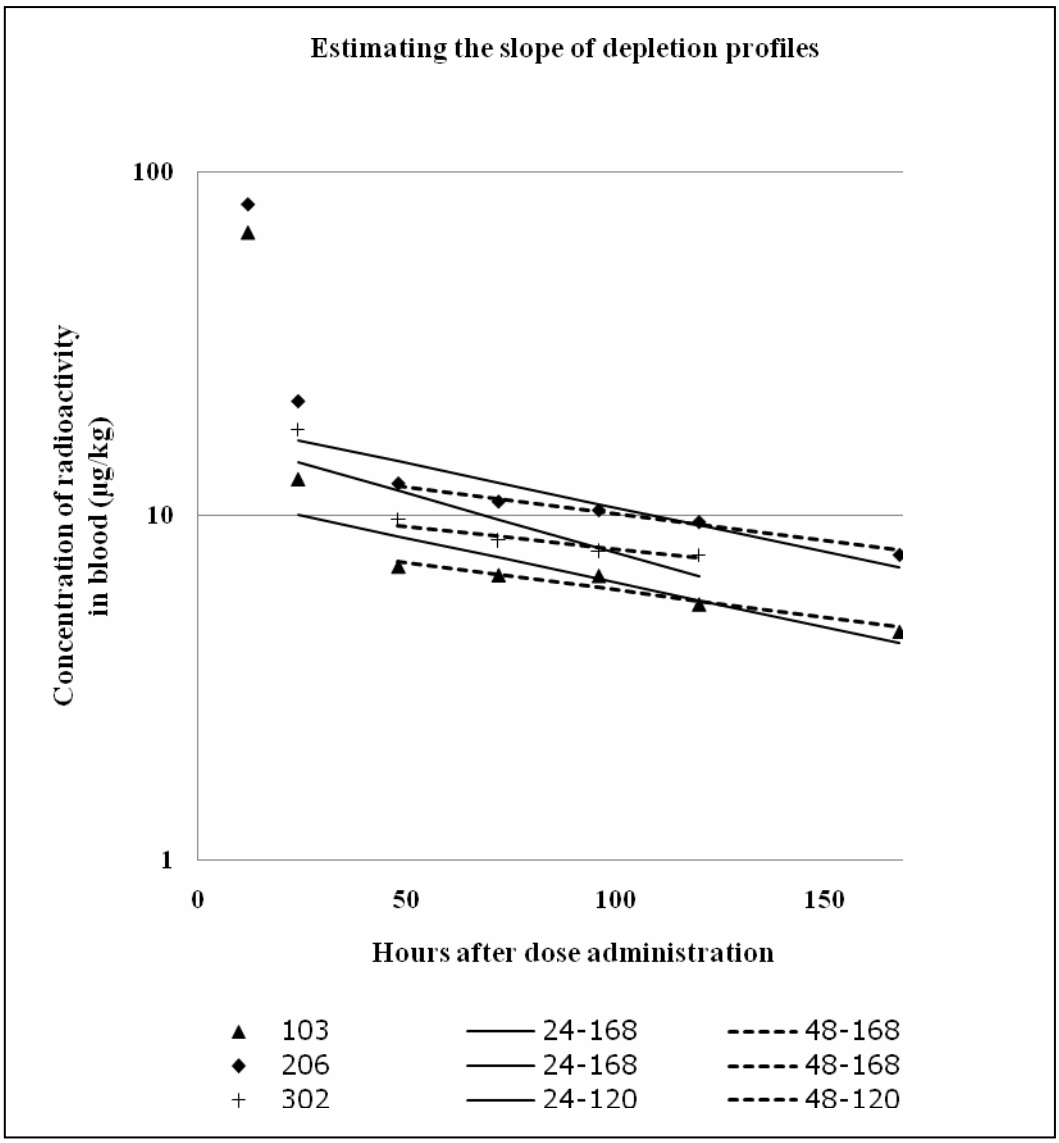
Rats receiving lyophilised tissues were allowed to eat the diet for 4 h before it was removed, weighed, and replaced with normal diet. Blood samples (150 µL) were taken 1, 2, 3, 4, 6, 9, 12, 24, 48, 72, 96 and 120 hours after initial exposure to the diet containing [^{14}C]-triclabendazole (Group C) 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

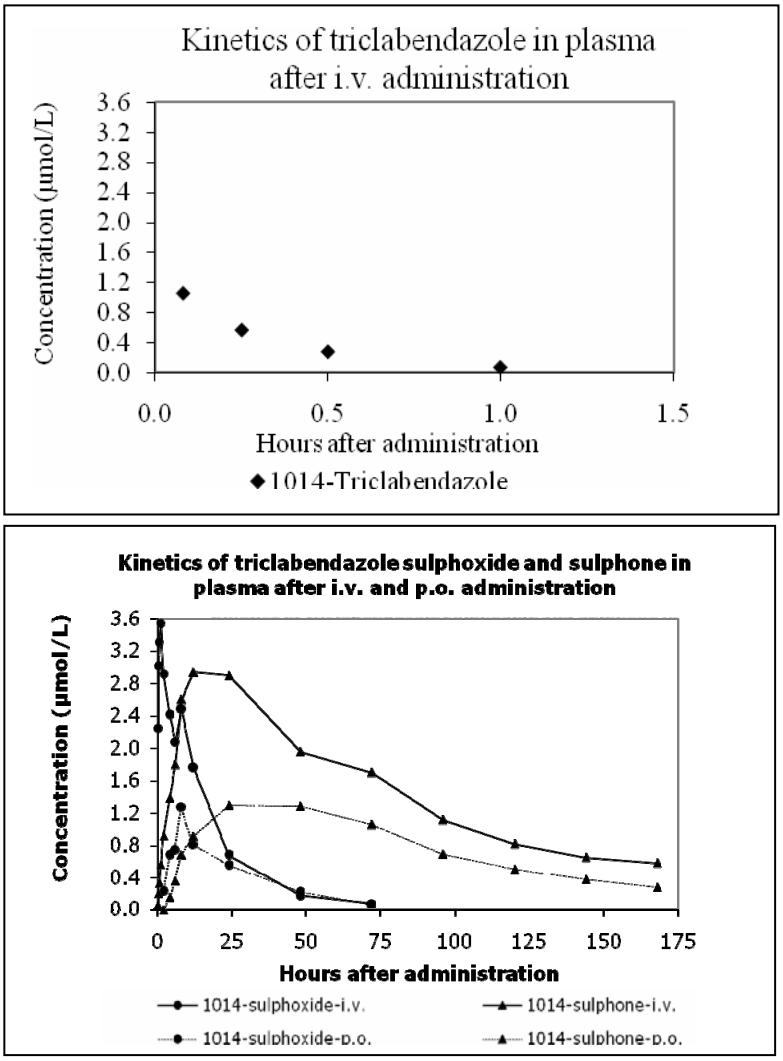
[^{14}C]-labelled triclabendazole was administered i.v. and p.o. to two female Chinchilla rabbits (Wiegand, et al., 1991a). The animals ranged from 2.7 to 4.3 kg over the duration of the study. The doses were administered at intervals of at least 4 weeks, first with an i.v. dose of 3 mg/kg of bw, then 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

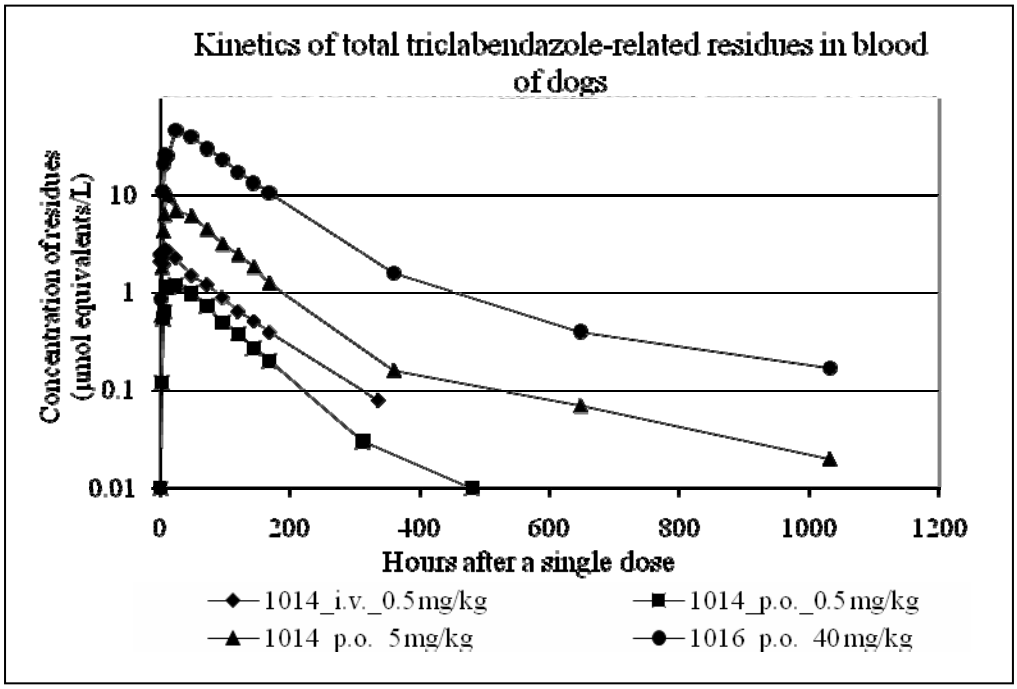
A large study in dogs and rats was conducted that investigated the absorption, distribution and excretion of [^{14}C]-triclabendazole (Schütz, et al., 1991). The concentrations of triclabendazole and its sulphoxide and sulphone metabolites in plasma and urine of dogs and rats, following i.v. and p.o. 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

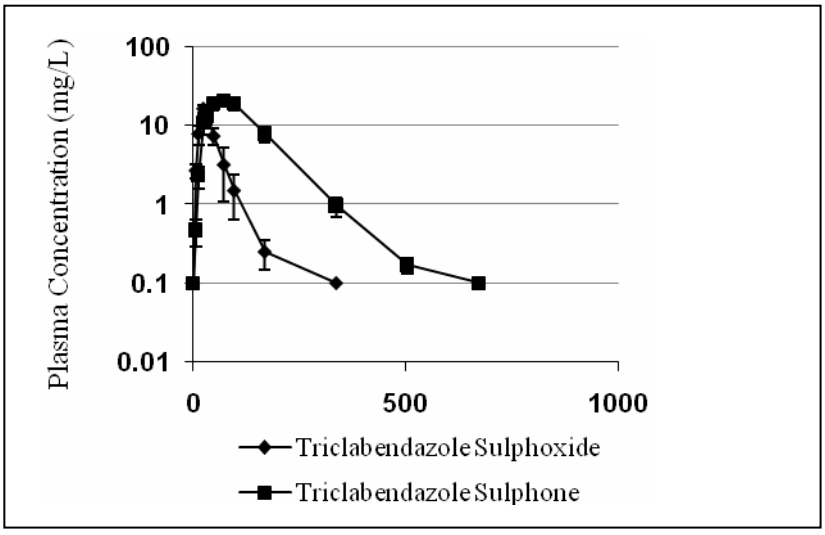
A fractionation study (Needham, 2004b) showed that 92-98.6% of the radioactivity in liver and muscle was associated with either lipid or protein. Changing the pH of the extracting solvent from acidic to basic did not release the tissue residues, suggesting that they are covalently bound to macromolecules 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.

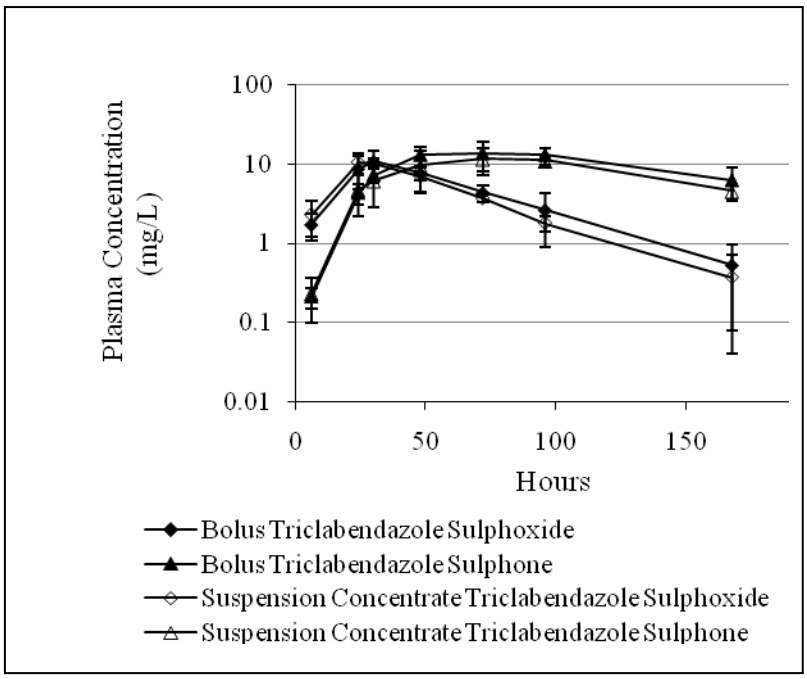
Four calves weighing 165-196 kg were administered an oral dose of 12 mg of triclabendazole (as a 10% w/v suspension)/kg of bw (Bull, et al., 1986a). Plasma samples were taken and analysed by HPLC for triclabendazole sulphoxide and triclabendazole sulphone. The semi-logarithmic plots of metabolite plasma 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.

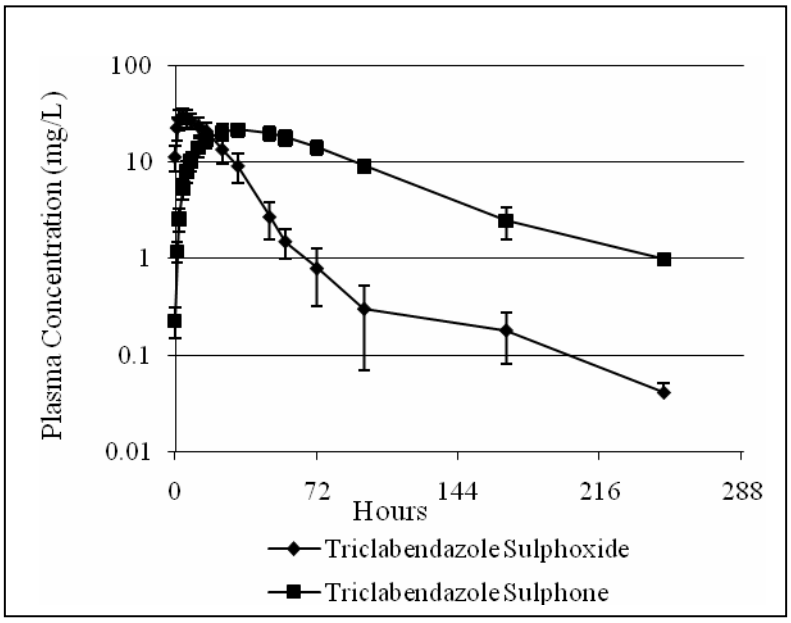
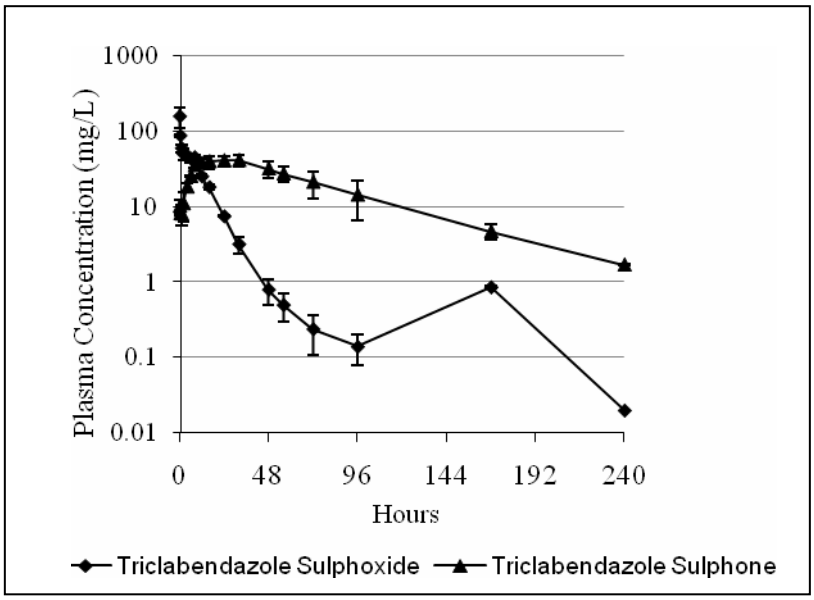


Figure 12: Plasma concentration of triclabendazole sulphoxide and triclabendazole sulphone *versus* time after i.v. administration of 12 mg triclabendazole sulphoxide/kg of bw to cattle.



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

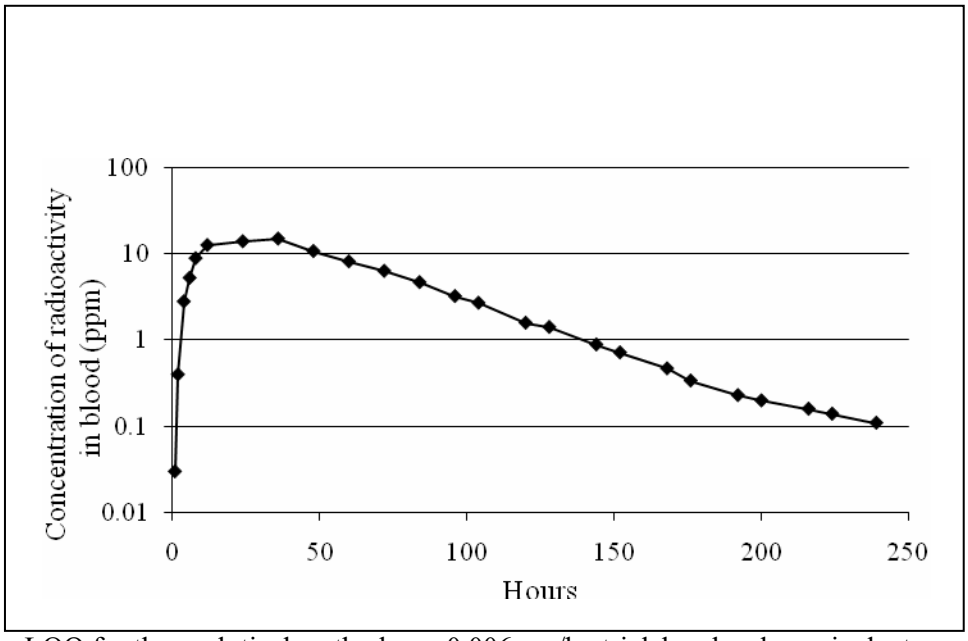
[^{14}C]–CGA-89 317 (specific radioactivity of 1.129 MBq/mg and radiochemical purity of 99%) was administered orally in gelatine capsules at a rate of 10.5 mg/kg to a 4 months old female sheep (Swiss White Alp X Ile de France breed), that weighed 28.5 kg (Hamböck and Strittmatter, 1982). Blood samples 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.

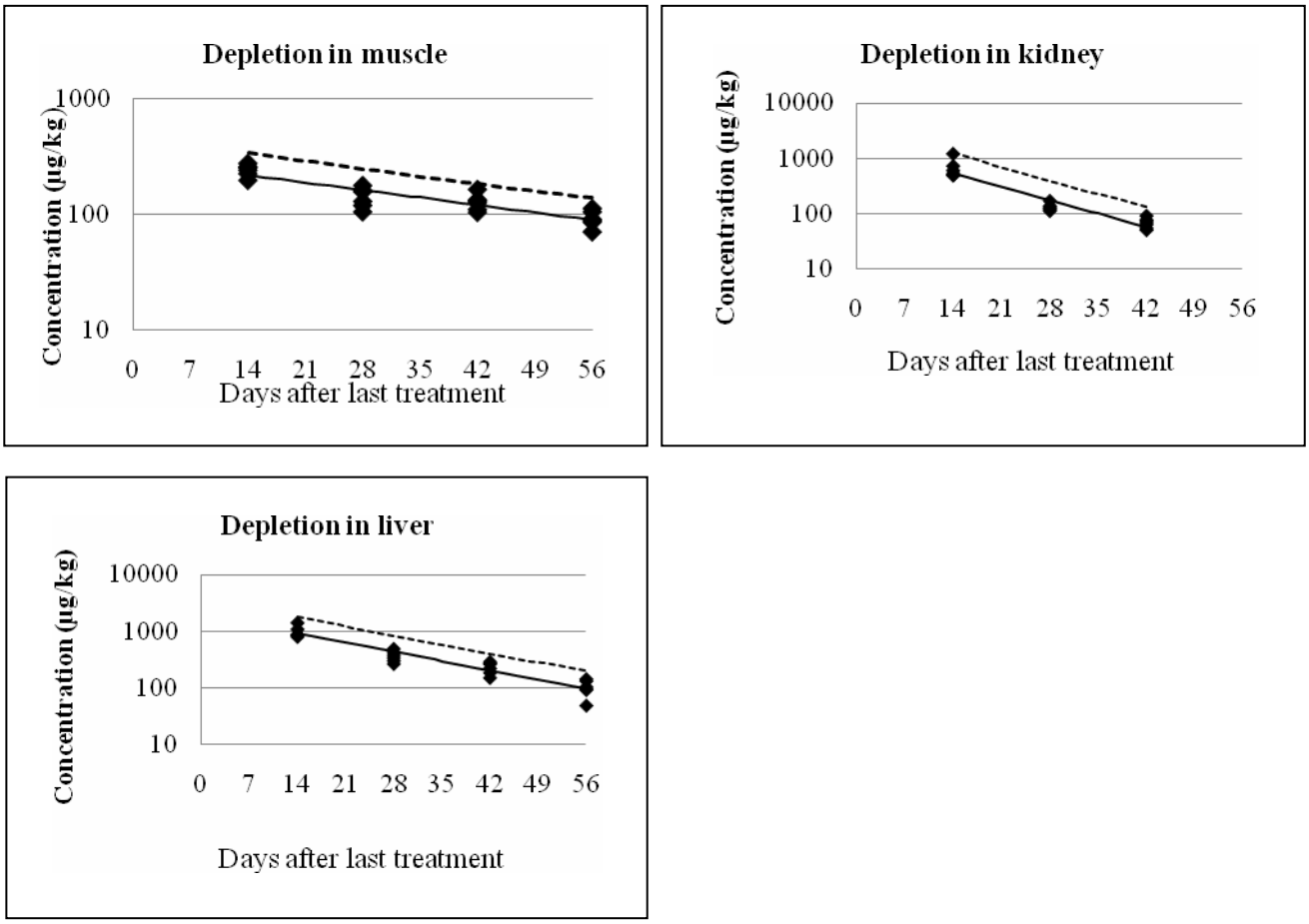
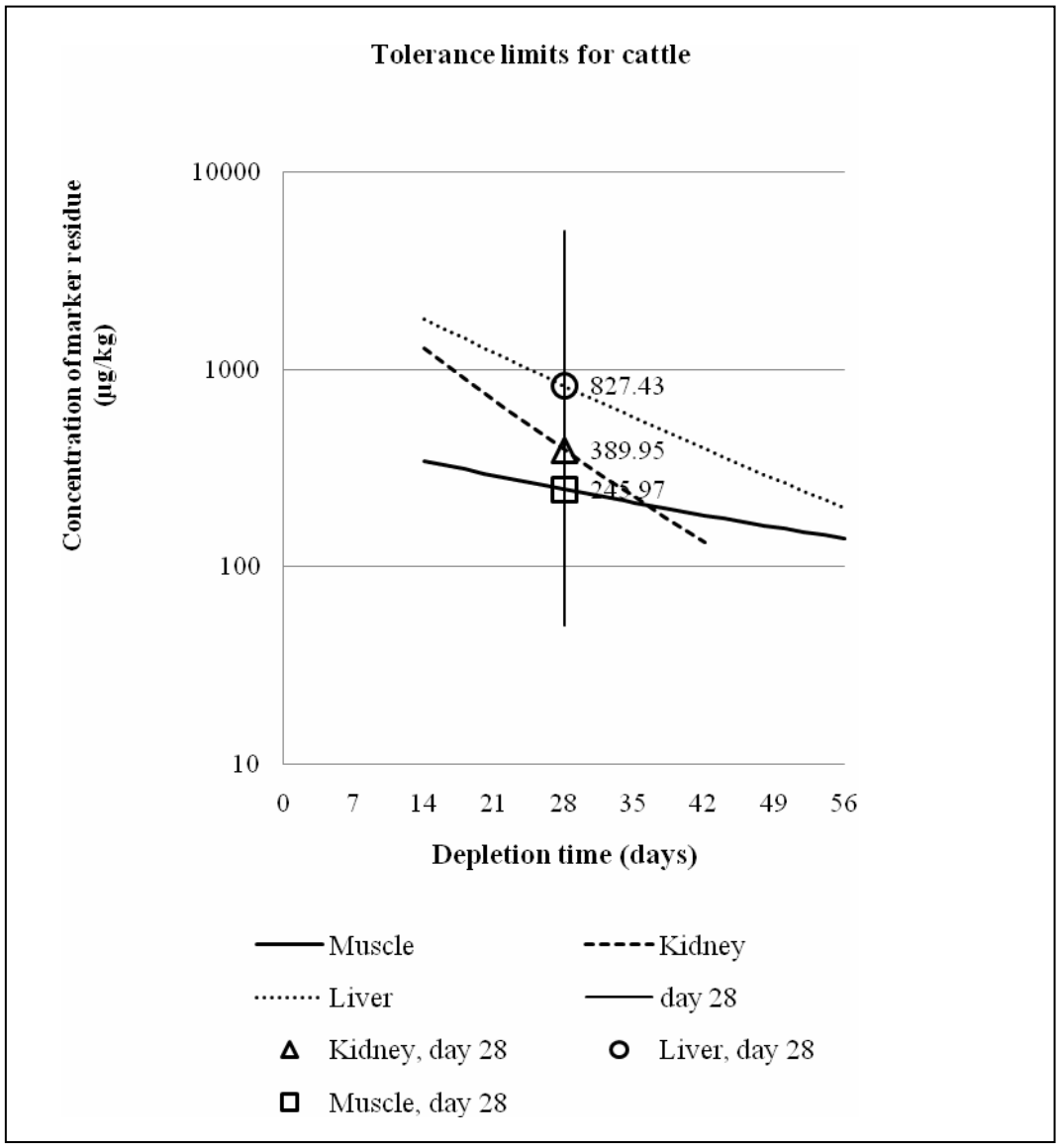


Figure 15 shows only the tolerance limit curves for the concentration of marker residue in liver, kidney and muscle of cattle. It also highlights the corresponding values for day 28, the only day when the ratios of the concentrations of marker and total residue are known. This is the only day for which dietary intake estimates are possible.

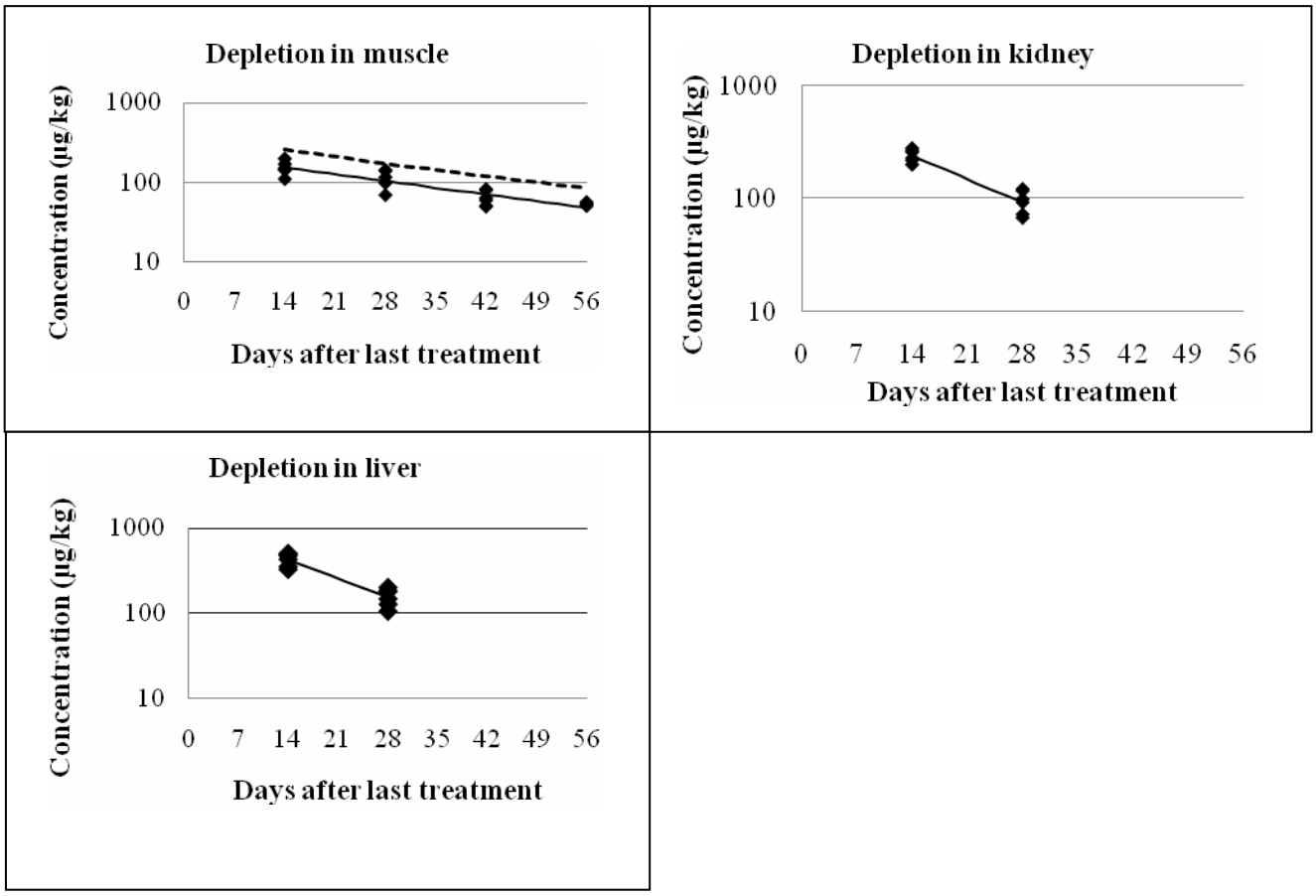
Figure 15: Tolerance limit curves for the concentration of marker residue in tissues of cattle and the concentration of marker residue in these tissues at day 28.



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.

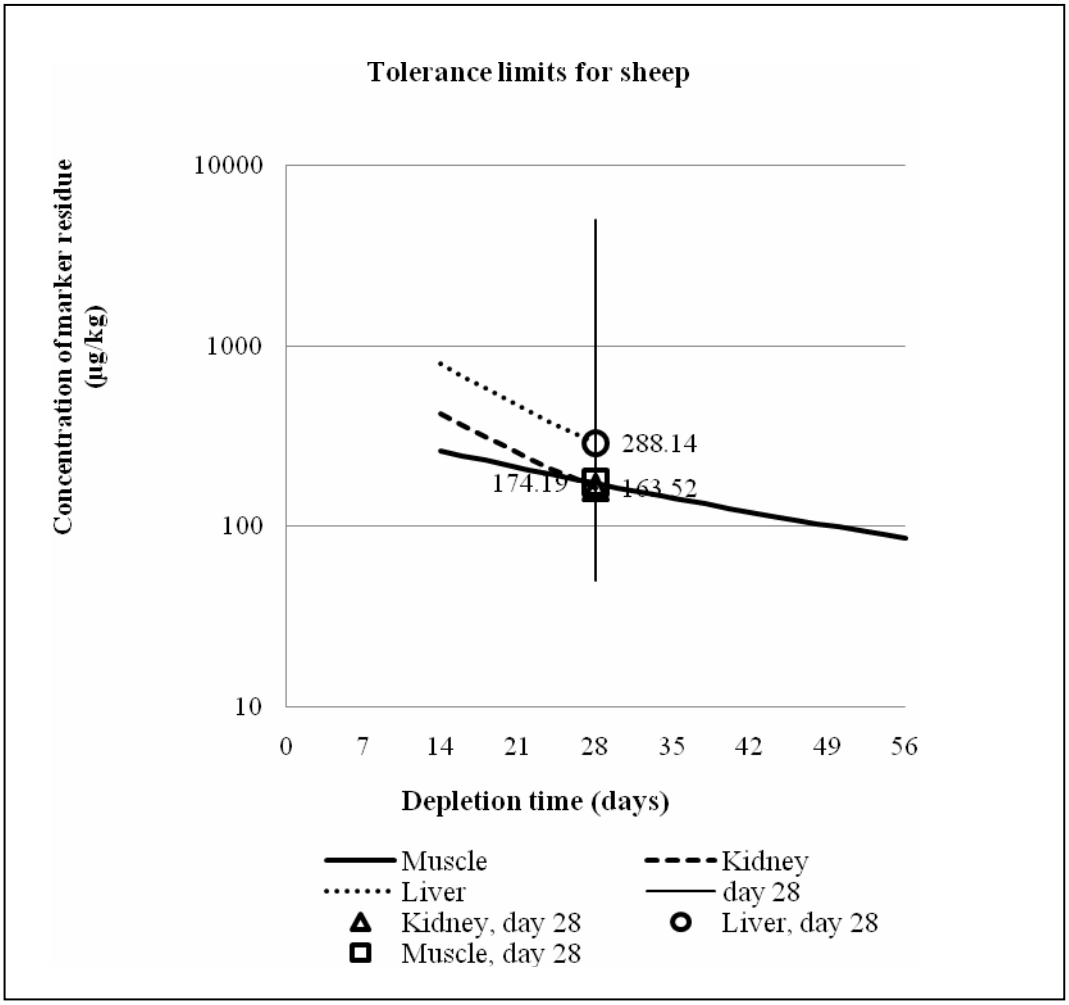
Similar considerations to those described above for cattle were applied to the evaluation of the sheep residue data. Figure 16 shows the depletion of marker residue in tissues of sheep.

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.

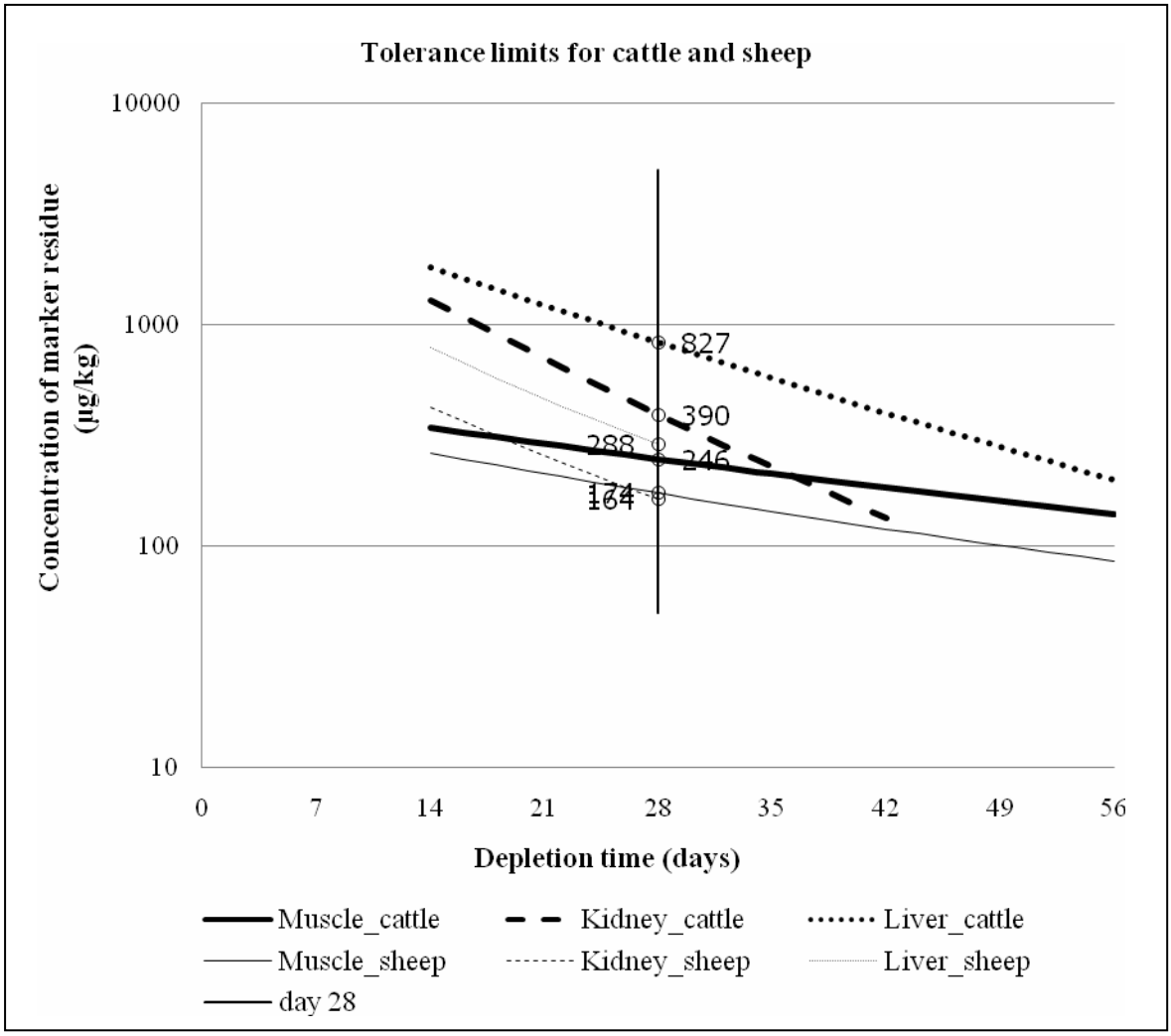


The proportions of the tolerance limits calculated for day 28 are 288:164:174 for liver:kidney:muscle. Table 23 shows calculations from the modelling of MRLs in sheep.

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.

REFERENCES

Adams, S. (2004a). Tissue residues of triclabendazole, measured as CGA 110754, in sheep following oral dosing with Fasinex 5%. Novartis Animal Health Australasia Pty Ltd, Report No. 04/07/1894, Study Y04/22.

Adams, S. (2004b). Validation of analytical procedure No. 193.F00. Tissue residues of triclabendazole, measured as CGA 110754, in cattle following repeated oral dosing with Fasinex 10%. Novartis Animal Health Australasia Pty Ltd., Report No. 04/02/1875, Study Y03/49.

Adams, S. (2004c). Validation of analytical procedure No. 193.F00. Determination of residues of triclabendazole in animal tissues by HPLC. Novartis Animal Health Australasia Pty Ltd, Report No. TR 04/05/1886, Study No. V03/57.

Adams, S. (2004d). Validation of analytical procedure No. 193.F00 Addendum 1. Stability of incurred residues of triclabendazole in cattle tissues under freezer storage conditions. Novartis Animal Health Australasia Pty Ltd, Report No. TR 04/07/1895, Study Plan No. V03/57.

Adams, S. (2004e). Validation of analytical procedure No. 193F.00 for goat tissues. Novartis Animal Health Australasia Pty Ltd, Report No. TR 04/09/1901, Study Y04/51.

Adams, S. (2004f). Addendum 1. Tissue residues of triclabendazole, measured as CGA 110754, in sheep following oral dosing with Fasinex 5%. Stability of incurred residues of triclabendazole in sheep tissues under freezer storage conditions. Novartis Animal Health Australasia Pty Ltd, Report No. TR 04/09/1902, Study Y04/22.

Adams, S. (2005). Extended validation of analytical procedure 193F.00 for sheep and cattle tissues. Study No V05/24. Novartis Animal Health Australasia Pty Ltd, Report No. TR05/06/1945.

Bull, M.S., Strong, M.B., and Kearney, E.M. (1986a). The persistence of the sulphoxide and sulphone metabolites in the plasma of cattle following oral dosage with triclabendazole at 12 mg a.i./kg. Ciba-Geigy Australia Limited, Technical Memorandum No. M86/12/1103.

Bull, M.S., Strong, M.B., and Kearney, E.M. (1986b). The bioavailability of triclabendazole and its sulphoxide metabolite in cattle. Ciba-Geigy Australia Limited, Technical Report No. 86/12/1099.

Bull, M.S., Bowen, F.L., and Malone, T. (1990). Pharmacokinetics of Fasinex 1800 Boli in cattle (Y90/11). Ciba-Geigy Australia Limited, Technical Report No. 90/5/1291.

Dieterle, R., and Kissling, M. (1995). Validation of Method REM 15/83: Determination of common moiety CGA 110754 in muscle, liver, kidney and fat of cattle as well as in muscle and liver of sheep after administration of ^{14}C-CGA 89317 by High Performance Liquid Chromatography (HPLC). Ciba-Geigy, Report on special study 132/94.

Downs, J.H., Marsh, J.D., and Krautter, G.R. (1991). Depletion of total drug-related residues in tissues of beef cattle treated with [^{14}C] triclabendazole (pilot study). Ciba-Geigy Ltd, Report No. 1375.

EMEA (1996). Note for guidance: Approach towards harmonisation of withdrawal periods. European Medicines Agency/Committee for Veterinary Medicinal Products, EMEA/CVMP/036/95-FINAL. Available at the website of EMEA at: http://www.emea.europa.eu/pdfs/vet/swp/003695en.pdf (Accessed 11 February 2009).

FAO (1993). Residues of some veterinary drugs in animals and foods. FAO Food and Nutrition Paper No. 41/5.

FAO/WHO (1993). Evaluation of Certain Veterinary Drug Residues in Foods (Fortieth Report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series No. 832.

FAO/WHO (2006). Evaluation of Certain Veterinary Drug Residues in Animals and Foods (Sixty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 939.

Ferguson, E.G.W. (1994a). [^{14}C]-89317: Absorption, distribution and excretion following a single oral administration of Fasinex – 10% to ruminating cattle. Hazleton Europe, Report No. 380/214-1011.

Ferguson, E.G.W. (1994b). [^{14}C]-CGA 89317: Absorption, distribution and excretion following a single oral administration of Fasinex-5% to ruminating sheep. Hazleton Europe, Report No. 380/215-1011.

Giannone, C. (1983). Determination of total residues in tissues and fat of sheep and cattle; REM 3-38. Ciba-Geigy Limited.

Giannone, C., and Formica, G. (1983). Determination of total residues in animal tissues and fat; REM 15/83. Ciba-Geigy Limited.

Hamböck, H. (1982). Characterization of tissue residues of CGA 89317 in sheep and goat. Ciba-Geigy Ltd, Project Report 50/82.

Hamböck. H. (1983). The metabolic fate of CGA 89317 in sheep, rat and the lactating goat. Ciba-Geigy Limited, Project Report No. 41/83.

Hamböck. H., and Strittmatter, J. (1981). Distribution, degradation and excretion of CGA 89317 in the lactating goat. Ciba-Geigy Limited, Project Report 34-81.

Hamböck. H., and Strittmatter, J. (1982). Distribution, degradation and excretion of CGA 89317 in sheep. Ciba-Geigy Australia Limited, Project Report 10/82.

Hardwick, T. (2004a). [^{14}C]-Triclabendazole: Absorption, distribution, excretion and metabolism in the rat. Covance Laboratories Ltd., Report No. 1969/024-D1145.

Hardwick, T. (2004b). [^{14}C]-Triclabendazole: Bioavailability of total residues in bovine tissues to rats. Covance Laboratories Ltd., Covance Report No. 1969/025-D1145.

Hassler, S. (1995). Bioavailability study in rats fed with CGA 89317 related tissue residues derived from cattle and sheep. Ciba-Geigy Ltd, Project Report 6/95.

Hennessy, D.R., Lacy, E., Steel, J.W., and Prichard, R.K. (1987). The kinetics of triclabendazole disposition in sheep. J. vet. Pharmacol. Therap. 10, 61-72.

Krautter, G.R. (1992). The characterization of [^{14}C] triclabendazole-related residues in tissues from a cow treated with a single dose of [^{14}C] triclabendazole. Ciba-Geigy Ltd, Report No. 1375.

Mohammed Ali, N.A.K., Bogan, J.A., Marriner, S.E., and Richards, R.J. (1986). Pharmacokinetics of triclabendazole alone or in combination with fenbendazole in sheep. J. vet. Pharmacol. Therap. 9, 442-445.

Muecke, W. (1981). Distribution, degradation and excretion of CGA 89317 in the rat. Ciba-Geigy Ltd., Project Report No. 27/81.

Needham, D. (2004a). [^{14}C]-Triclabendazole: Pharmacokinetics in the rat following oral and intravenous administration. Covance Laboratories Ltd., Report No. 1969/026-D1145.

Needham, D. (2004b). [^{14}C]-Triclabendazole: Distribution and excretion following oral administration to cattle. Covance Laboratories Ltd, Covance Report No. 1969/023-D1145.

Sanyal, P.K. (1994). Pharmacokinetic study of triclabendazole in sheep and goat using a high performance liquid chromatography method. Indian Journal of Pharmacology; 22: 200-203.

Schütz, H., Lecaillon, J. B., and Godbillon, J. (1991). Kinetics of unchanged CGP 23030 and its sulphoxide and sulphone metabolites in plasma and urine of dogs, and in urine of rats following

intravenous and oral administration of single doses of [^{14}C]-labelled CGP 23030. Laboratoire Ciba-Geigy, Biopharmaceutical Research Centre, Report CRBR 25/1991.

Schütz, H., Cudd, A., and Probst, A. (1996). [^{14}C]-CGP 23 030: Absorption and distribution studies in rats and dogs. Ciba-Geigy Ltd, Report No. DM23/1991.

Strehlau, G.A. (2004a). Study Y03/49. Residue of keto-triclabendazole in cattle tissues following repeated oral dosing with Fasinex 10%. Report on statistical evaluation, 4 May 2004. Novartis Animal Health Inc.

Strehlau, G.A. (2004b). Study Y04/22. Residues of keto-triclabendazole in sheep tissues following oral dosing with Fasinex 5%. Report on statistical evaluation, 27 August 2004, Novartis Animal Health Inc.

Strong, M.B., Bull, M.S., and Shume, G. (1982). Pharmacokinetic studies carried out with CGA-89317 and its metabolites CGA-110752 and CGA-110753 in sheep. Ciba-Geigy Australia Ltd, Technical Report No. 82/7/916.

Strong, M.B., Bull, M.S., Shume, G., and Kearney, E.M. (1983). Pharmacokinetics of CGA-89317: Influence of (a) individual sheep, (b) oesophageal groove reflex, and (c) formulations, on the plasma levels of parent and metabolite compounds. Ciba-Geigy Australia Ltd., Technical Report No. 83/3/948.

Strong, M.B., Bull, M.S., Shume, G.E., and Ochudzawa, Z. (1987). Studies to determine the plasma profiles of the metabolites of triclabendazole and of levamisole in cattle following the treatment of Fasinex, Nilverm or a combination formulation of the two active ingredients. Ciba-Geigy Australia Limited, Technical Report No. 87/9/1151.

Strong, M.B., Bull, M.S., Shume, G.E., and Malone, T. (1988). Studies to determine the plasma profiles of the metabolites of triclabendazole and of levamisole in sheep following the treatment of Fasinex, Nilverm or a combination formulation of the two active ingredients. Ciba-Geigy Australia Ltd., Technical Report No 88/1/1178.

Study No. AA031 (2001). Determination of the tissue residues following treatment of cattle with an abamectin/triclabendazole pour-on formulation. Sponsor: M. Forster, Ancare NZ Limited. Study Director: B. Chick, Veterinary Health Research Pty Limited, West Armidale, Australia. Laboratory: Amdel New Zealand Limited, Auckland, NZ.

Study No. ANT1274 (2002). Determination of the tissue residues in beef cattle following administration of an abamectin and triclabendazole pour-on formulation. Sponsor: M. McArthur, Ancare New Zealand Limited. Study Director: M. Chambers, Veterinary Health Research Pty Limited, West Armidale, Australia. Laboratory: D. Hennessy, VHR Analytical Laboratory, North Ryde, Australia.

Study No EL-55021 (2004). Determination of abamectin/triclabendazole tissue residues in beef cattle after treatment with GENESIS ULTRA POUR-ON. Estendart Limited, Massey University, Palmerston North, New Zealand.

Thanei, P. (1995a). The metabolite profiles in urine and faeces extract of ruminating cattle after administration of [U-^{14}C]benz-imidazole CGA 89317. Ciba-Geigy Ltd, Project Report 2/95.

Thanei, P. (1995b). The metabolite profiles in urine and faeces extract of ruminating sheep after administration of [U-^{14}C] benzimidazole CGA 89317. Ciba-Geigy Ltd, Project 017 AM03.

Wiegand, H., Schütz, H., and Probst, A. (1991a). CGP 23 030, Triclabendazole, Fasinex. Absorption and distribution studies in female rabbits. Ciba-Geigy Ltd, Report No. DM 12/1991.

Wiegand, H., Schütz, H., Lecaillon, J. B., and Godbillon, J. (1991b). Kinetics of unchanged CGP 23030 and its sulphoxide and sulphone metabolites in rabbits following intravenous and oral administration of single doses of [^{14}C]-labelled CGP 23 030. Ciba-Geigy Laboratories, Report No. CRBR 22/1991.

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

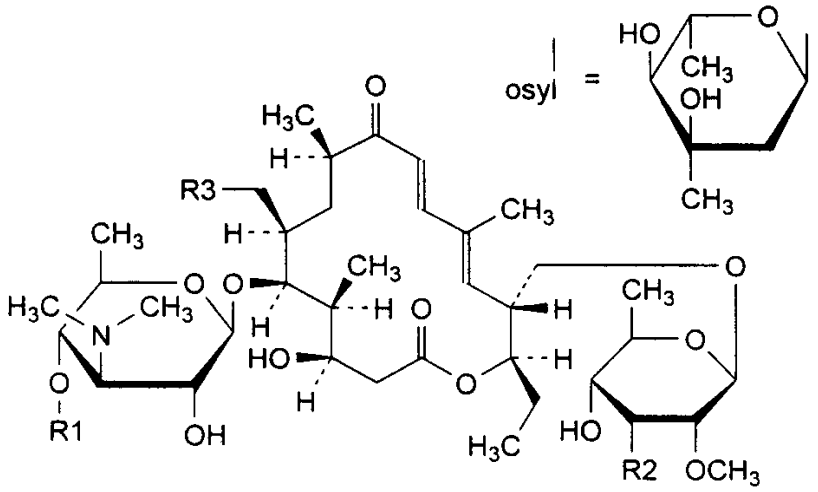
Chemical Abstracts System number: CAS 1401-69-0

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).

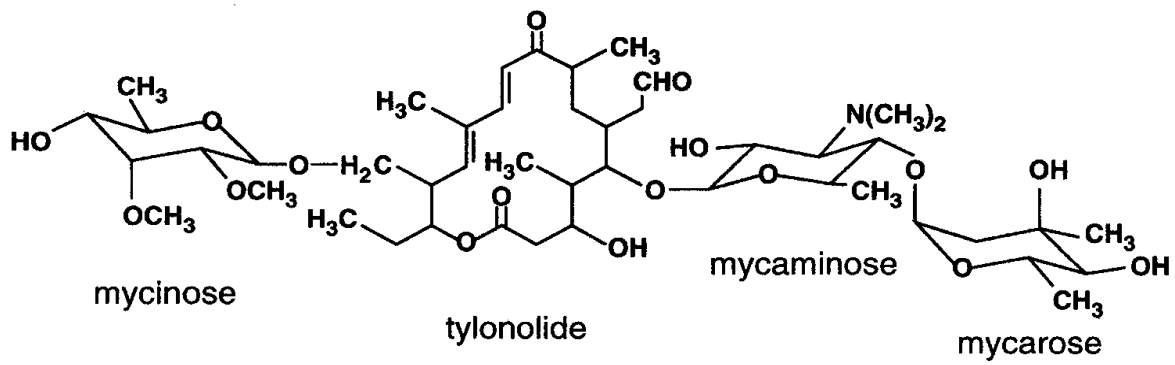
Figure 1: Chemical structure of tylosin.



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

REFERENCES

Abdul-Karim, B.G. (2006a). Study No. T1G1010402. Pre-clinical laboratory study: pharmacokinetics of tylosin in cattle and calves. Elanco Animal Health, A Division of Eli Lilly and Company, Greenfield, IN, USA. Sponsor submitted.

Abdul-Karim, B.G. (2006b). Study No. T1G1010403. Pre-clinical laboratory study: pharmacokinetics of tylosin in chicken. Elanco Animal Health, A Division of Eli Lilly and Company, Greenfield, IN, USA. Sponsor submitted.

Adam, G., Ferguson, L., and MacDougall, J. (2007). Validation of an analytical method for the determination of tylosin in chicken eggs. Study No. 210410. Charles River Laboratories, Tranent, Edinburgh, UK. Sponsor submitted.

Anderson, R.C., Worth, H.M., Small, R.M., and Harris, P.N. (1966). Toxicological studies on tylosin: its safety as a food additive. Food Cosmet. Toxicol., 4, 1-15.

Atef, M., Youssef, S.A.H., Atta, A.H., and El-Maaz, A.A. (1991). Disposition of tylosin in goats. Br. Vet. J., 147, 207-215.

Baggot, J.D. (1978). Some aspects of clinical pharmacokinetics in veterinary medicine: principles of pharmacokinetics. J. Vet. Pharmacol. Ther., 1, 111-118.

Baggot, J.D., and Gingerich, D.A. (1976). Pharmacokinetic interpretation of erythromycin and tylosin activity in serum after intravenous administration of a single dose to cows. Res. Vet. Sci., 21, 318-323.

Baltz, R.H., and Seno, E.T. (1988). Genetics of Streptomyces fradiae and tylosin biosynthesis. Annu. Rev. Microbiol., 42, 547-574.

Baltz, R.H., Seno, E.T., Stonesifer, J., and Wild, G.M. (1983). Biosynthesis of the macrolide antibiotic tylosin. A preferred pathway from tylactone to tylosin. J. Antibiot., 36, 131-141.

Benetti, C., Dainese, N., Biancotto, G., Piro, R., and Mutinelli, F. (2004). Unauthorised antibiotic treatments in beekeeping. Development and validation of a method to quantify and confirm tylosin residues in honey using liquid chromatography-tandem mass spectrometric detection. Anal. Chim. Acta, 520, 87-92.

Burnett, T.J., Marth, J.L., Fossler, S.C., and Kiehl, D.E. (1999). ^{14}C-tylosin metabolism and residue decline in laying hens administered medicated drinking water: part 1 of 2: analysis and characterization of radioactive residues in eggs. Unpublished GLP study No. T1Y729901 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Sponsor submitted.

Burrows, G.E. (1980). Pharmacotherapeutics of macrolides, lincomycins, and spectinomycin. J. Am. Vet. Med. Assoc., 176, 1072-1077.

Burrows, G.E., Barto, P.B., Martin, B. and Tripp, M.L. (1983). Comparative pharmacokinetics of antibiotics in newborn calves: chloramphenicol, lincomycin, and tylosin. Am. J. Vet. Res., 44, 1053-1057.

Burrows, G.E., Barto, P.B., and Martin, B. (1986). Antibiotic disposition in experimental pneumonic pasteurellosis: gentamicin and tylosin. Can. J. Vet. Res. 50, 193-199.

Cacciapuoti, A.F., Loebenberg, D., Moss, E.L., Jr., Menzel, F.W., Jr., Rudeen, J.A., Naples, L.R., Cramer, C.L., Hare, R.S., Mallmas, A.K., and Miller, G.H. (1990). Microbiological and pharmacokinetic studies of acyl demycinosyltylosin and related tylosin derivatives. J. Antibiot. (Tokyo), 43, 1131-1136.

Caldow, M., Stead, S.L., Day, J., Sharman, M., Situ, C., and Elliott, C. (2005). Development and validation of an optical SPR biosensor assay for tylosin residues in honey. J. Agric. Food Chem., 53, 7367-7370.

Carter, K.K., Hietala, S., Brooks, D.L., and Baggot, J.D. (1987). Tylosin concentrations in rat serum and lung tissue after administration in drinking water. Lab. Anim. Sci., 37, 468-470.

Cester, C.C., Ganiere, J.P., and Toutain, P.L. (1993). Effect of stage of oestrous cycle on tylosin disposition in genital tract secretions of cows. Res. Vet. Sci., 54, 32-39.

Cochrane, R.L., and Thomson, T.D. (1990). The determination of tylosin residues in lung and serum following administration of Tylan® 200 by intramuscular injection to pigs. Unpublished study T1Z769002 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Sponsor submitted.

Curtis, M.A. (1999). Tylosin phosphate in feed for dairy cows. Milk residue study – final report. Unpublished study T1XAL9801 from Elanco Animal Health, West Ryde, Australia and Bovine Strategic Services, Camden, Australia. Sponsor submitted.

Dudriková, E., and Lehotský, J. (1998). A comparison of liquid chromatographic and microtitre test procedures for determining the depletion of tylosin in healthy lactating cows. Milchwissenschaft, 53, 90-92.

Duthu, G.S. (1985). Interspecies correlation of the pharmacokinetics of erythromycin, oleandomycin, and tylosin. J. Pharm. Sci., 74, 943-946.

El-Sayed, M.G.A., El-Attar, H.M., Atef, M., and Yousif, M. (1986). Pharmacokinetic profile of tylosin in mastitic cows. Dtsch. Tierärztl. Wschr., 93, 326-328.

EMEA (1996). Note for guidance: Approach towards harmonisation of withdrawal periods. European Medicines Agency/Committee for Veterinary Medicinal Products, EMEA/CVMP/036/95-FINAL. Available at the website of EMEA at: http://www.emea.europa.eu/pdfs/vet/swp/003695en.pdf (Accessed 11 February 2009).

European Pharmacopoeia 5.0 (2004). Tylosin for veterinary use. Directorate for the Quality of Medicines of the Council of Europe, Council of Europe, Strasbourg, vol. 2, pp. 2647-2648.

FAO (1991). Residues of some veterinary drugs in animals and foods. FAO Food and Nutrition Paper 41/4.

FAO (2000). Procedures for recommending maximum residue limits - residues of veterinary drugs in food (1987-1999). Available at the FAO JECFA website at: ftp://ftp.fao.org/es/esn/jecfa/2000-06-30_JECFA_Procedures_MRLVD.pdf (Accessed 11 February 2009).

FAO/WHO (1969). Specifications for the Identity and Purity of Food Additives and their Toxicological Evaluation: Some antibiotics (Twelfth Report of the Joint FAO/WHO Expert Committee on Food Additives), FAO Nutrition Meetings Report Series No. 45; WHO Technical Report Series No. 430.

FAO/WHO (1991). Evaluation of Certain Veterinary Drug Residues in Foods (Thirty-Eighth Report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series No. 815.

FAO/WHO (2006). Evaluation of Certain Veterinary Drug Residues in Animals and Foods (Sixty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 939.

FDA (2005). Tylan (tylosin tartrate) soluble. In: NADA 013-076. Freedom of Information Summary, Supplemental New Animal Drug Application, pp. 1-12. Available at the website of the Center for Veterinary Medicine, US Food and Drug Administration, at: http://www.fda.gov/cvm/foi/013-076s101705.pdf (Accessed 10 August, 2008).

Feldlaufer, M.F., Kochansky, J.P., Pettis, J.S., and Vincent, D. (2006). Potential residues of tylosin tartrate in honey. In: Tylan® Soluble (tylosin tartrate) for the control of American foulbrood (Paenibacillus larvae) in honeybees. International Summary Dossier, Elanco Animal Health. Sponsor submitted.

Furusawa, N. (2001). Transference of dietary veterinary drugs into eggs. Vet Res. Commun., 25, 651-662.

García-Mayor, M.A., Garcinuño, R.M., Fernández-Hernando, P., and Durand-Alegría, J.S. (2006). Liquid chromatography-UV diode array detection method for multi-residue determination of macrolide antibiotics in sheep's milk. J. Chromatogr. A, 1122, 76-83.

Gaynor, M., and Mankin, A.S. (2005). Macrolide antibiotics: binding site, mechanism of action, resistance. Front. Med. Chem., 2, 21-35.

Giguère, S. (2006). Macrolides, azalides, and ketolides. In: Antimicrobial Therapy in Veterinary Medicine (4th edition), ed. Giguère S., Prescott J.F., Baggot J.D., Dowling P.M., Blackwell Publishing, pp. 191-205.

Gingerich, D.A., Baggot, J.D., and Kowalski, J.J. (1977). Tylosin antimicrobial activity and pharmacokinetics in cows. Can. Vet. J., 18, 96-100.

González de la Huebra, M.J., Vincent, U., and von Holst, C. (2007). Sample preparation strategy for the simultaneous determination of macrolide antibiotics in animal feedingstuffs by liquid chromatography with electrochemical detection (HPLC-ECD). J. Pharm. Biomed. Anal., 43, 1628-1637.

Grassetti, A., and Villa, S. (2001a). Tylan® Sulpha premix tissue residue in swine. Unpublished GLP study No. T5UPIT010 from Research Toxicology Centre S.p.A, Pomezia (Roma), Italy. Sponsor submitted.

Grassetti, A., and Villa, S. (2001b). Tylan® G 250 premix egg residue in laying hens. Unpublished GLP study No. T1XAIT001 from Eli Lilly Italia S.o.A., Sesto Fiorentino, Italy. Sponsor submitted.

Hamill, R.L. and Stark, W.M. (1964). Macrocin, a new antibiotic, and lactenocin, an active degradation product. J. Antibiot. (Tokyo), 17, 133-139.

Hamill, R.L., Haney, M.E., Jr., Stamper, M., and Wiley, P.F. (1961). Tylosin, a new antibiotic. II. Isolation, properties, and preparation of desmycosin, a microbiologically active degradation product. Antibiot. Chemother., 11, 328-334.

Hammel, Y.-A., Mohamed, R., Gremaud, E., LeBreton, M.-H., and Guy, P.A. (2008). Multi-screening approach to monitor and quantify 42 antibiotic residues in honey by liquid chromatography-tandem mass spectrometry. J. Chromatogr. A, 1177, 58-76.

Hamscher, G., Limsuwan, S., Tansakul, N., and Kietzmann, M. (2006). Quantitative analysis of tylosin in eggs by high performance liquid chromatography with electrospray ionization tandem mass spectrometry: residue depletion kinetics after administration via feed and drinking water in laying hens. J Agric. Food Chem., 54, 9017-9023.

Hjerpe, C.A. (1979). A comparison of serum antibiotic concentrations achieved in calves with intratracheal administration of procaine penicillin G, ampicillin trihydrate, tylosin, oxytetracycline hydrochloride, chloramphenicol sodium succinate, dihydrostreptomycin sulfate and neomycin sulfate

with those achieved with intravenous, intramuscular and subcutaneous administration. Bovine Pract., 14, 18-26.

Hu, D., and Coats, J.R. (2007). Aerobic degradation and photolysis of tylosin in water and soil. Environ. Toxicol. Chem., 26, 884-889.

Hu, D., Fulton, B., Henderson, K., and Coats, J. (2008). Identification of tylosin photoreaction products and comparison of ELISA and HPLC methods for their detection in water. Environ. Sci. Technol., 42, 2982-2987.

Iritani, Y., Hidaka, S., Kitabatake, T., and Ise, T. (1975). Tylosin levels in the tissue of pig after medication in drinking water. Jap. J. Zootech. Sci., 48, 588-590.

Iveković, D., Lopotar, N., Brajša, K., and Mandić, Z. (2003). Electrochemical reduction of desmycosin, structure investigation and antibacterial evaluation of the resulting products. Eur. J. Pharmac. Sci., 18, 323-328.

Kan, C.A., and Petz, M. (2000). Residues of veterinary drugs in eggs and their distribution between yolk and white. J. Agric. Food Chem., 48, 6397-6403.

Kennington, A.S., and Donoho, A.L. (1994). ^{14}C-tylosin rat metabolism study. Unpublished GLP studies T1X759102 and R25091 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Sponsor submitted.

Kennington, A.S., Donoho, A.L., Darby, J.M., Moran, J.W., and Occolowitz, J.L. (1994a). Tylosin metabolism study in tissues and excreta of calves injected with 14C-tylosin. Unpublished GLP study No. T1Z749302 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Sponsor submitted.

Kennington, A.S., Donoho, A.L., Darby, J.M., Moran, J.W., and Occolowitz, J.L. (1994b). Tylosin metabolism in tissues and excreta of pigs dosed with ^{14}C-tylosin. Unpublished GLP studies No. T1X759101 and T1X749303 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Sponsor submitted.

Keukens, H.J. (1996). A study in dairy cows: Residues in milk after repeated intramuscular administration of Tylan® 200 injectable. Unpublished GLP study No. RIK.HKE.96.02 (TY1R016) from the Institute of Veterinary Research, Wezep for Elanco Animal Health, The Netherlands. Sponsor submitted.

Kietzmann, M. (1985). Comparative study of the distribution and excretion of tylosin of various origins. Dtsch. Tierarztl. Wochenschr., 92, 147-149.

Kim, M.-H., Gebru, E., Chang, Z.-Q., Choi, J.-Y., Hwang, M.-H., Kang, E.-H., Lim, J.-H., Yun, H.-I., and Park, S.-C. (2008). Comparative pharmacokinetics of tylosin or florfenicol after a single intramuscular administration at two different doses of tylosin-florfenicol combination in pigs. J. Vet. Med. Sci., 70, 99-102.

King, N., and Walker, A. (2007). Determination of tylosin residues in chicken eggs following the administration of 500 mg tylosin/litre in the drinking water for 5 consecutive days. Study No. 284189. Charles River Laboratories, Tranent, Edinburgh, UK. Sponsor submitted.

Kiorpes, A.L. (1993). Relative availability of Tylan® 200 Injection administered subcutaneously and intramuscularly to cattle. Unpublished GLP study No. HWI 6180-110 from Hazelton Wisconsin Inc., Madison, Wisconsin. Sponsor submitted.

Kochansky, J. (2004). Degradation of tylosin residues in honey. J. Apicult. Res., 43, 65-68.

Kowalski, C., Roliński, Z., Zań, R., and Wawron, W. (2002). Pharmacokinetics of tylosin in broiler chickens. Pol. J. Vet. Sci., 5, 127-130.

Lacoste, E. (2003). Pharmacokinetic study of tylosin in broiler chicken using an aqueous solution (via the intravenous route and oral gavage) and as a feed slurry (via oral gavage). Unpublished GLP study No. T1YAFR0202 from Avogadro, Parc de Génibrat, Fontenilles, France. Sponsor submitted.

Lewicki, J. (2006). Tylosin. A review of pharmacokinetics, residues in food animals and analytical methods. Available at the website of FAO at: ftp://ftp.fao.org/ag/agn/food/tylosin_2006.pdf (Accessed 10 August 2008).

Litterio, N.J., Calvinho, L.F., Flores, M.M., Tarabla, H.D., and Boggio, J.C. (2007). Microbiological screening test validation for detection of tylosin excretion in milk of cows with low and high somatic cell counts. J. Vet. Med. A, 54, 30-35.

Luperi, L., and Villa, S. (1999). Tylan® 200 Injection tissue residue study in dairy cattle. Unpublished study No. 6956 (T1XCIT9801) from the Research Toxicology Centre, S.p.A, Pomezia (Roma), Italy. Sponsor submitted.

Marth, J.L., Burnett, T.J., Kiehl, D.E., and Buck, J.M. (2001). ^{14}C-tylosin metabolism and residue decline in broiler chickens administered medicated drinking water. Unpublished GLP study No. T1Y720001 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Sponsor submitted.

Marth, J.L., Burnett, T.J., Kiehl, D.E., Da, D.H., and Fossler, S.C. (2000). ^{14}C-tylosin metabolism and residue decline in laying hens administered medicated drinking water: part 2 of 2: analysis and characterization of radioactive residues in tissues and excreta. Unpublished GLP study No. T1Y729901 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Sponsor submitted.

Matsuoka, T., and Johnson, W.S. (1976). Milk residue studies in lactating cows following intramuscular injection of tylosin. Unpublished study No. 659-G128-10 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Sponsor submitted.

McFarland, J.W., Berger, C.M., Froshauer, S.A., Hayashi, S.F., Hecker, S.J., Jaynes, B.H., Jefson, M.R., Kamicker, B.J., Lipinski, C.A., Lundy, K.M., Reese, C.P., and Vu, C.B. (1997). Quantitative structure-activity relationships among macrolide antibacterial agents: in vitro and in vivo potency against Pasteurella multocida. J. Med. Chem., 40, 1340-1346.

McGuire, J.M., Boniece, W.S., Higgens, C.E., Hoehn, M.M., Stark, W.M., Westhead, J., and Wolfe, R.N. (1961). Tylosin, a new antibiotic, I. Microbiological studies. Antibiot. Chemother., 11, 320-327.

McReynolds, J.L., Caldwell, D.Y., McElroy, A.P., Hargis, B.M., and Caldwell, D.J. (2000). Antimicrobial residue detection in chicken yolk samples following administration to egg-producing chickens and effects of residue detection on competitive exclusion culture (PREEMPT) establishment. J. Agric. Food Chem., 48, 6435-6438.

Mertz, J.L., and Graper, L.K. (1982). ^{14}C-tylosin tissue residue study in swine. Unpublished GLP study No. ABC-0016 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Sponsor submitted.

Montesissa, C., De Liguoro, M., Santi, A., Capolongo, F., and Biancotto, G. (1999). Tylosin depletion in edible tissues of turkeys. Food Add. Contam., 16, 405-410.

Moran, J.W., Coleman, M.R., Thomson, T.D., and Cochrane, R.L. (1990). The determination of tylosin residue in milk following administration of Tylan® 200 by intramuscular injection to dairy cows. Unpublished GLP study No. T1Z709003 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Sponsor submitted.

Nagy, J., Popelka, P., Sokol, J., Turek, P., and Neuschl, J. (2001). The excretion of tylosin residues in ewes' milk after its experimental administration. Folia Vet., 45, 196-198.

Nalda, M.J.N., Yagüe, J.L.B., Gómez, M.T.M., Sevilla, J.J.J., del Nozal, J.B., and Pascual, M.H. (2006). Trace analysis of antibacterial tylosin A, B, C and D in honey by liquid chromatography-electrospray ionization-mass spectrometry. J. Sep. Sci., 29, 405-413.

Narandja, A., Kelnerić, Z., Kolacny-Babić, L., and Djokić, S. (1995). 10,11,12,13-Tetrahydro derivatives of tylosin. II. Synthesis, antibacterial activity and tissue of 4'-deoxy-10,11,12,13-tetrahydrodesmycosin. J. Antibiot. (Tokyo)., 48, 248-253.

Nouws, J.F.M., and Ziv, G. (1977a). The persistence of antibiotic residues at the intramuscular injection site of dairy cows. Ref. Vet., 34, 131-135.

Nouws, J.F.M., and Ziv, G. (1977b). Tissue distribution and residues of tylosin in normal and emergency-slaughtered dairy cows and calves. Arch. Lebensmittelhyg., 28, 92-94.

Nouws, J.F.M., and Ziv, G. (1979). Distribution and residues of macrolide antibiotics in normal dairy cows. Arch. Lebensmittelhyg., 30, 202-208.

Paesen, J., Cypers, W., Pauwels, E., Roets, J., and Hoogmartens, J. (1995a). Study of the stability of tylosin A in aqueous solutions. J. Pharmac. Biomed. Anal., 13, 1153-1159.

Paesen, J., Claeys, P., Cypers, W., Roets, J., and Hoogmartens, J. (1995b). Liquid chromatography of tylosin A and related substances on poly(styrene-divinylbenzene). J. Chromatogr. A, 699, 93-97.

Paesen, J., Cypers, W., Busson, R., Roets, J., and Hoogmartens, J. (1995c). Isolation of decomposition products of tylosin using liquid chromatography. J. Chromatogr. A, 699, 99-106.

Peng, Z., and Bang-Ce, Y. (2006). Small molecule microarrays for drug residue detection in foodstuffs. J. Agric. Food Chem., 54, 6978-6983.

Plumb, D.C. (2002). Tylosin. In: Veterinary Drug Handbook (4th edition), Iowa State Press a Blackwell Publishing Company, pp. 821-823.

Poulsen, S.M., Kofoed, C., and Vester, B. (2000). Inhibition of the ribosomal peptidyl transferase reaction by the mycarose moiety of the antibiotics carbomycin, spiramycin and tylosin. J. Mol. Biol., 304, 471-481.

Prats, C., El Korchi, G., Francesch, R., Arboix, M., and Perez, B. (2002a). Disposition kinetics of tylosin administered intravenously and intramuscularly to pigs. Res. Vet. Sci., 73, 141-144.

Prats, C., El Korchi, G., Francesch, R., Arboix, M., and Perez, B. (2002b). Tylosin depletion from edible pig tissues. Res. Vet. Sci., 73, 323-325.

Retsema, J., and Fu, W. (2001). Macrolides: structures and microbial targets. Int. J. Antimicrob. Agents, 18, S3-S10.

Roberts, S. (2007). Validation of an analytical method for the determination of tylosin in chicken liver, kidney, muscle, skin with fat and eggs. Analytical Method No. 1610. Study No. 211608, Charles River Laboratories, Tranent, Edinburgh, UK. Sponsor submitted.

Roudaut, B., and Moretain, J.P. (1990). Residues of macrolide antibiotics in eggs following medication of laying hens. Br. Poult. Sci., 31, 661-675.

Sauter, R.A., Corbe, H.T., and Bailey, R.W. (1962). Blood level studies in the bovine, equine and porcine species with tylosin, a new antibiotic. J. Vet. Med., 57, 982-986.

Sieck, R.F., Graper, L.K., Giera, D.D., Herberg, R.J., and Hamill, R.L. (1978a). Metabolism of tylosin in swine and rat. Unpublished study from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Sponsor submitted.

Sieck, R.F., Graper, L.K., Giera, D.D., Herberg, R.J., and Hamill, R.L. (1978b). ^{14}C-tylosin residue study in swine. Unpublished study from Agricultural Biochemistry, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Sponsor submitted.

Sokol, J., Matisová, E., Dudriková, E., and Cabadaj, R. (1996). Determination of tylosin in milk by HPLC using SPE. Toxicol. Lett., 88, (Suppl. 1), 95-96. Abstract P3R-345.

Song, W., Huang, M., Rumbeiha, W., and Li, H. (2007). Determination of amprolium, carbadox, monansin, and tylosin in surface water by liquid chromatography/tandem mass spectrometry. Rapid Commun. Mass Spectrom., 21, 1944-1950.

Tang, H.P., Ho, C., and Lai, S.S. (2006). High-throughput screening for multi-class veterinary drug residues in animal muscle using liquid chromatography/tandem mass spectrometry with on-line solid-phase extraction. Rapid Commun. Mass Spectrom., 20, 2565-2572.

Teeter, J.S., and Meyerhoff, R.D. (2003). Aerobic degradation of tylosin in cattle, chicken, and swine excreta. Environ. Res., 93, 45-51.

Thomson, T.D., and Moran, J.W. (1994). Tylosin kidney and liver residues in cattle 21 days following 8 mg/lb of Tylan® 200 Injection for 5 consecutive days by intramuscular or subcutaneous injection. Unpublished study No. T1Z769101 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Sponsor submitted.

Thompson, T.S., Noot, D.K., Calvert, J., and Pernal, S.F. (2003). Determination of lincomycin and tylosin residues in honey using solid-phase extraction and liquid chromatography-atmospheric pressure chemical ionization mass spectrometry. J. Chromatogr. A, 1020, 241-250.

Thompson, T.S., Noot, D.K., Calvert, J., and Pernal, S.F. (2005). Determination of lincomycin and tylosin residues in honey by liquid chromatography/tandem mass spectrometry. Rapid Commun. Mass Spectrom., 19, 309-316.

Thompson, T.S., Pernal, S.F., Noot, D.K., Melathopoulos, A.P., and van den Heever, J.P. (2007). Degradation of incurred tylosin to desmycosin – implications for residue analysis of honey. Anal. Chim. Acta, 586, 304-311.

van Duyn, R.L. (Undated). Tylosin serum and lung tissue concentrations following intramuscular administration of swine. Unpublished study No. 766-G125-81 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Sponsor submitted.

van Duyn, R.L., and Folkerts, T.M. (1979). Concentrations of tylosin in blood and lung tissue from calves given single and repeated daily intramuscular doses. Vet. Med. Small Anim. Clin., 74, 375-377.

van Duyn, R.L., and Johnson,W.S. (Undated). Tylosin levels in serum and lung tissue from swine injected intramuscularly once with tylosin. Unpublished study No. 766-G125-72 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Sponsor submitted.

van Duyn, R.L., and Kline, R.M. (Undated-a). Tylocine® Injection intramuscular canine blood levels. Unpublished Pharmacology study No. VPR-199-766 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Sponsor submitted.

van Duyn, R.L., and Kline, R.M. (Undated-b). Additional studies on the pharmacology and toxicology of Tylocine® Injection in dogs and cats. Unpublished pharmacology study from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Sponsor submitted.

van Duyn, R.L., Kline, R.M., and Russell, E. (Undated). Tylocine® intramuscular canine blood levels. Unpublished pharmacology study No. VPR-139-766 from Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN, USA. Sponsor submitted.

Vincent, U., Gizzi, G., von Holst, C., de Jong, J., and Michard, J. (2007). Validation of an analytical method for the determination of spiramycin, virginiamycin and tylosin in feeding-stuffs by thin-layer chromatography and bio-autography. Food Add. Contam., 24, 351-359.

Walker, A., Rae, A., and Roberts, S. (2007). Residue depletion of tylosin in broiler chickens following administration of Tylan® Soluble in the drinking water for five consecutive days at a level of 500 mg tylosin/litre of water. Study No. 284681, Charles River Laboratories, Tranent, Edinburgh, UK. Sponsor submitted.

Wang, J. (2004). Determination of five macrolide antibiotic residues in honey by LC-ESI-MS and LC-ESI-MS/MS. J. Agric. Food Chem., 52, 171-181.

Wang, J., Leung, D., and Lenz, S.P. (2006). Determination of five macrolide antibiotic residues in raw milk using liquid chromatography-electrospray ionization tandem mass spectrometry. J. Agric. Food Chem., 54, 2873-2880.

Warren, M.J. (1998). Tylosin residue depletion study in chicken eggs following oral administration via the drinking water. Unpublished GLP study No. TYL-98-01 from the Biological Services Unit, The Royal Veterinary College, University of London, Hatfield, Herts, UK. Sponsor submitted.

Weisel, M.K., Powers, J.D., Powers, T.E., and Baggot, J.D. (1977). A pharmacokinetic analysis of tylosin in the normal dog. Am. J. Vet. Res., 38, 273-275.

Whaley, H.A., Patterson, E.L., Dornbush, A.C., Backus, E.J., and Bohonos, N. (1963). Isolation and characterization of relomycin, a new antibiotic. Antimicrob. Agents Chemother. 161, 45-48.

WHO (1991). Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives, Series 29.

Wilson, R.C. (1984). Macrolides in veterinary medicine. In: Macrolide Antibiotics. Chemistry, Biology, and Practice (1st edition), ed. Omura S., Academic Press, Inc., pp. 301-347.

Yoshida, M., Hoshii, H., Yonezawa, S., Nakamura, H., and Yamaoka, R. (1973). Residue of dietary tylosin in blood, muscle and liver of growing chicks. Jap. Poult. Sci., 10, 23-28.

Zhanel, G.G., Dueck, M., Hoban, D.J., Vercaigne, L.M., Embil, J.M., Gin, A.S., and Karlowsky, J.A. (2001). Review of macrolides and ketolides. Focus on respiratory tract infections. Drugs, 61, 443-498.

Ziv, G. (1980). Preliminary clinical pharmacological investigations of tylosin and tiamulin in chickens. Vet. Quarterly, 2, 206-210.

Ziv, G., and Risenberg, R (1991). Oral bioequivalence studies of two tylosin products in broilers and layers. Prakt. Tierärzt., 72, 860-863.

Ziv, G., and Sulman, F.G. (1973). Serum and milk concentrations of spectinomycin and tylosin in cows and ewes. Am. J. Vet. Res., 34, 329-333.