MELENGESTROL ACETATE*

First draft prepared by

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ADDENDUM

To the monograph and its addendum prepared by the 54th and 58th meetings of the Committee and published in FAO Food and Nutrition Papers 41/13 and 41/14, respectively.

INTRODUCTION

Melengestrol acetate (17α-acetoxy-6-methyl-16-methylenepregna-4,6-diene-3,20-dione acetate; MGA) is a progestogen that is used as an animal feed additive to improve feed efficiency, increase the rate of weight gain, and suppress oestrus in beef heifers. MGA is fed at daily doses of 0.25 – 0.50 mg per heifer for 90 to 150 days prior to slaughter. The Committee at its 54th meeting (Annex 1, reference 146) recommended temporary MRLs of 5 μg/kg for cattle fat and 2 μg/kg for cattle liver, and requested information on an analytical method suitable for quantifying residues of MGA in liver and fat tissue (JECFA, 2000). At its 58th meeting (Annex 1, reference 157), the Committee concluded that the analytical method submitted for evaluation had been validated for monitoring compliance with the MRLs, and recommended that the temporary MRLs for cattle liver and fat be made permanent (JECFA, 2002).

At its 54th meeting, the Committee was provided with insufficient information to characterise the structure and activity of the metabolites of MGA. The Committee therefore assumed that the metabolites were equipotent to MGA in terms of progestogenic activity in elaborating the temporary MRLs. At its 62nd meeting the Committee considered new data on the metabolism of MGA in vitro , which provided the structural identities of the major metabolites of MGA, as well as a report describing the results of in vitro transcriptional activation/reporter assays , which were used to determine the relative hormonal activities of MGA and its metabolites.

METABOLISM

The extensive metabolism of MGA in several animal species and in humans was documented in previous reports. In the present studies, the metabolic profile of MGA was characterized by means of the generation and isolation of metabolites in test systems in vitro, since the concentrations of metabolites in tissues and excreta from cattle fed with MGA were too low for this purpose. The test systems investigated used hepatic microsomes, hepatic S9 fractions, and liver slices, all of which were prepared from beef heifers. The metabolites were separated by semi-preparative HPLC and their structures characterized by HPLC, HPLC-MS and nuclear magnetic resonance (NMR).

Preliminary in vitro experiments were conducted to optimise the conditions for generating the greatest relative yield of metabolites. Typically, microsomes (0.5 mg/mL protein) or S9 fractions (1 mg/mL protein) were incubated at 37oC with the desired concentration of MGA and 1 mM NADPH. Reactions were terminated by the addition of ice-cold acetonitrile and the samples centrifuged at approximately 1000 g for 10 min. The supernatants were recovered and analysed by HPLC.

Incubation time for the in vitro generation of metabolites was optimised using batched liver microsomes prepared from several heifers. Pooled microsomes (0.5 mg/mL microsomal protein) were incubated with 100 μM MGA and 1 mM NADPH for 0, 1, 3, 5, 10, 20, 30, 60, and 120 min. Recovered supernatants were analysed by HPLC-UV. Based on the number and quantities of metabolites produced, an incubation time of 120 min was selected for experiments designed to generate metabolites. Metabolites were labelled A through E, according to the order in which they eluted on chromatography. The conversion of MGA and the formation of metabolites in liver microsomes prepared from heifers are given in Table 1. The trace amounts of Metabolite A generated were insufficient to quantify or characterise this metabolite.

Table 1 Conversion of MGA and formation of metabolites in bovine liver microsomes incubated for 120 minutes.

MGA converted (%)	Metabolite B formed (%)	Metabolite C formed (%)	Metabolite D formed (%)	Metabolite E formed (%)
25	1	5	2	15

* During the editing of the monograph for melengestrol acetate (MGA) some inconsistencies in the approach to derive the activity weighing factors for MGA-related residues where detected which could be corrected partially. To address all of them requires a revision at the next meeting of JECFA that will assess residues of veterinary drugs.

The effect of MGA concentration on the metabolite profiles was investigated in a separate experiment. Pooled liver microsomes from heifers were incubated for 30 min with MGA at 1, 12.4, 31, and 100 μM with at least 3 replicates per concentration. Sufficient amounts of Metabolites B, C, D, and E for quantification were generated at a concentration of 100 μM MGA (Table 2), the concentration chosen for subsequent experiments.

Table 2 Conversion of MGA and formation of metabolites in bovine liver microsomes incubated for 30 minutes.

MGA conc μM	MGA converted (%)	Metabolite B formed (%)	Metabolite C formed (%)	Metabolite D formed (%)	Metabolite E formed (%)
1	65	9	35	0	50
100	10	<2	20	12	65

The metabolite profiles of MGA from S9 fractions and liver microsomes from several beef cattle were compared. S9 fractions prepared from the livers of several male and female beef cattle were pooled and mixed in approximately equal sized batches for heifers and steers. Metabolites C and E were the most abundantly produced metabolites; Metabolite D was also formed. Other metabolites from the microsomal incubations were not observed in the S9 fractions.

A comparison of the metabolite profiles and examination for additional metabolites were undertaken in batches of mixed-sex bovine liver microsomes and from heifer-only liver microsomes. Similar metabolite profiles were generated for both batches and no new metabolites requiring characterisation were produced.

MGA metabolism was also investigated in liver slices prepared from two beef heifers. Metabolites C, D, and E, of which Metabolite C was the most abundant, was detected on HPLC analysis. At least three other peaks were present on the chromatograms, but were unrelated to MGA.

Additional studies with human microsomes, rat microsomes and human cytochrome P450 were performed to provide a better understanding of the historical data from comparative in vivo metabolism and toxicology studies of MGA.

A procedure similar to that described above for the bovine in vitro test systems was used with human and rat microsomes. Pooled microsomes from humans and rats were incubated with 100 μM MGA and 1 mM NADPH for 60 or 120 min. In human microsomes, Metabolite E was the most abundant metabolite, while significant quantities of Metabolites C, D, A, and B were produced. By comparison, in rat microsomes Metabolite C was the most abundant, Metabolites D and E were major metabolites, and Metabolites A and B were minor metabolites. Additional minor metabolites, identified by LC/MS as monohydroxy and dihydroxy metabolites, were produced by both human and rat microsomes but were present in insufficient quantities for further characterisation.

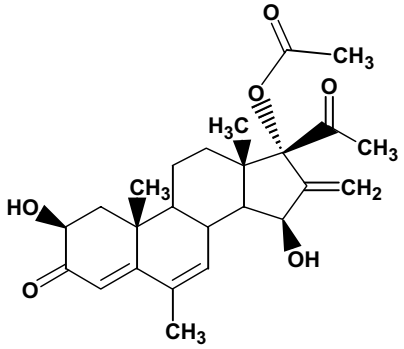
Human recombinant CYP450 isoenzymes, which were purchased as a pre-manufactured mixture and as individual isoenzymes of 1A2, 2C8, 2C9, 2C19, 2D6, and 3A4, were incubated under conditions identical to those described above. The results indicated that CYP450 metabolism of MGA is attributed primarily to 3A4. All major metabolites observed in human microsome incubations and in the isoenzyme mixture were produced by 3A4 in the same proportions, with Metabolite E being the most abundant metabolite. In contrast Metabolite C was the primary metabolite observed with 1A2, 2C8, 2C9, 2C19, and 2D6, with only a small amount of Metabolite E being produced.

MGA and its metabolites produced from the in vitro test systems were analysed by reverse phase HPLC with UV detection. Compounds were separated on a C-18 column and the eluants monitored by photodiode array detection at 285 nm. Different linear solvent gradient programs were used to analyse samples collected from liver microsomes and liver slices. A flow rate of 1 mL/min was used in both cases.

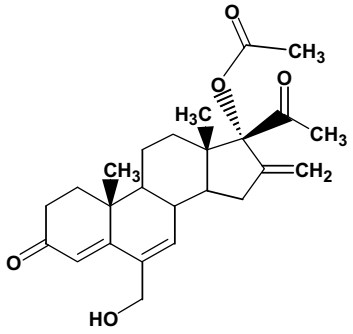
An experiment was undertaken to generate, isolate, and prepare MGA metabolites from heifer liver microsomes. Metabolites were generated using a 40x scale-up of the procedure described above for microsomes. Metabolite isolation was accomplished by reversed phase semi-preparative HPLC on a C18 column, a linear solvent gradient program, at a flow rate of 4 mL/min. Microsomal samples were loaded onto the column and 1-minute fractions collected. The MGA metabolites were then prepared for chemical structure characterisation.

The chemical structures of Metabolites B, C, D, and E were characterised using NMR and HPLC/MS; Metabolite A was not characterised since it was generated only in trace amounts. NMR data were acquired using a Varian INOVA 500 MHz NMR spectrometer operating at a proton observation frequency of 499.79 MHz and equipped with a Nalorac MIDTG 3-mm NMR probe. LC/MS analysis of Metabolites B, C, D, and E was performed on a ThermoFinnigan TSQ-Quantum triple quadrupole mass spectrometer operating in the positive-ion ESI mode. Separation by the LC/MS system was performed on a C18 column with a linear solvent gradient program at a flow rate of 1 mL/min. The eluant was monitored by photodiode array detection at 190-800 nm and MS detection of 150-900 amu.

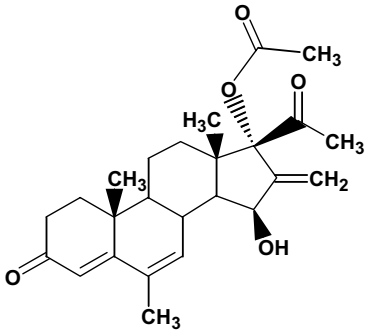
Structural assignments for the metabolites of MGA were based upon combined data from HPLC, LC/MS and NMR. Structures were assigned as follows:



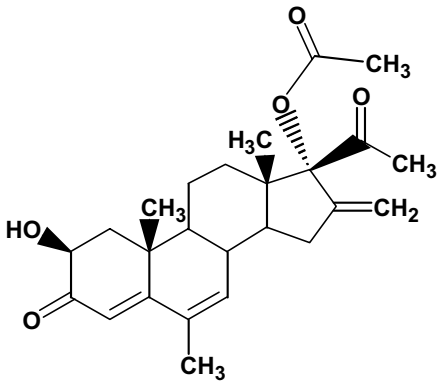
Metabolite B (2ß,15ß-dihydroxy-MGA)



Metabolite C (6-hydroxymethyl-MGA)



Metabolite D (15ß-hydroxy-MGA)



Metabolite E (2ß-hydroxy-MGA)

The metabolic pathway proposed for the biotransformation of MGA involved mono-hydroxylation of MGA to Metabolites C, D, and E. Metabolite B was presumed to form from Metabolite D (15β-hydroxy-MGA) by hydroxylation at C2, but not from Metabolite E (2β-hydroxy-MGA). This is consistent with the formation of Metabolite B in microsome incubations in which Metabolite D and not Metabolite E was isolated.

MGA metabolite steroid receptor specificity and relative potency

Samples of MGA, melengestrol, and Metabolites B, C, D, and E were prepared for testing by dilution in absolute ethanol to final concentrations of 100 μM for MGA and melengestrol, 1 μM for Metabolite B, and 10 μM for Metabolites C, D, and E. The purity of each metabolite, MGA and melengestrol as used in the in vitro transcriptional activator/reporter gene assay was determined to be >95% by HPLC-UV. Activity was compared against progesterone, R5020 (a synthetic progestin) and medroxyprogesterone acetate (MPA) in progesterone receptor assays; dexamethasone, hydrocortisone and medroxyprogesterone acetate in glucocorticoid receptor assays; dihydrotestosterone, R1881 (a synthetic androgen), progesterone and medroxyprogesterone acetate in androgen receptor assays; and 17β-estradiol, ethinyl estradiol (17α-ethinyl-17β-estradiol) and medroxyprogesterone acetate in oestrogen receptor assays. Comparator compounds were selected either because they exhibit hormonal activity in vivo due to their ability to act as agonists of specific hormone receptors, or they have close structural similarity to MGA. Melengestrol, while not detected as a metabolite in bovine metabolism studies, was included in this experiment since it is a potential enzymatic cleavage product of MGA.

In vitro transcriptional activation/reporter gene assays were used to determine the activities of compounds as agonists for the human progesterone receptor B-subtype (PR), the human glucocorticoid receptor (GR), the human androgen receptor (AR), and the human oestrogen receptor α-subtype (ERα). The B-subtype of the human progesterone receptor was chosen because it is the dominant subtype in humans. Monkey kidney CV-1 cells were transiently co-transfected with the designated human steroid receptor expression vector and a luciferase reporter vector containing the appropriate hormone response element. The mouse mammary tumour virus-luciferase reporter vector (MMTV-Luc) was used for PR, GR, and AR, whereas the oestrogen response element-luciferase reporter vector (ERE-Luc) was used for ERα. The response of each hormone receptor to test substance was determined by steroid receptor-mediated transcription of MMTV-Luc for PR, GR, and AR, or ERE-Luc for ERα, to produce luciferase and thus yield a measurable signal upon the introduction of luciferin.

Details of the PR, GR, AR, and ERα assay procedures were provided but are not discussed here. Each assay experiment was conducted over 3 days (ERα) or 4 days (PR, GR, and AR).

MGA, melengestrol, and Metabolites B, C, D, and E were evaluated in each hormone receptor assay. The test concentrations chosen for these experiments were consistent with the approach used in classic pharmacodynamic dose-response studies, in which the response is mathematically expressed by the standard Hill equation describing receptor-mediated responses, and the response is linearly related to the logarithm of concentration or dose (Novotny, 2001).

Each assay experiment was repeated three times in separate weeks for GR, AR, and ERα, and eight times for PR. Each test substance and each comparator compound was analysed in duplicate at each concentration. Control blanks comprising tissue culture media without steroid test substances and a pCMV5(p5) empty expression vector control were included. The latter lacked activity in the absence and presence of a control agonist, thereby demonstrating that transcriptional activity resulted from the expressed receptor and was independent of any receptor endogenous to CV1 cells.

The relative activity of each compound for each receptor was reported as the minimum effective concentration resulting in 50-100% maximal transactivation, as indicated by luciferase activity. Calculations used the lowest reported value in cases in which a range was reported. Where luciferase activity was not detectable (less than 5-fold higher than the no ligand control), the highest concentration tested that did not yield a response was used in calculations. In cases in which the highest

concentration tested produced a detectable response but did not result in 50-100% maximal transactivation, the highest tested concentration was used in calculations.

The response of MGA, its metabolites, and of the comparator compounds in the hormone receptor assays are shown in Table 4. The responses reported in this table represent approximate orders of magnitude of relative biological activity, with each value being the most common empirical observation for each ligand in each assay, without regard to statistical evaluation.

Table 4 Minimum effective concentrations (nM) for compounds to induce 50-100% maximal transactivation of human hormone receptors

Compound	PR	GR	AR	ERα
Progesterone	0.1	---	>100	---
Medroxyprogesterone acetate	0.01	10	1	>100
R5020	0.01	---	---	---
Dexamethasone	---	1	---	---
Cortisol	---	10	---	---
Dihydrotestosterone	---	---	0.1	---
R1881	---	---	0.1	---
17β-Estradiol	---	---	---	0.01
Ethinyl estradiol	---	---	---	0.01
MGA	0.01	1	>100	>100
Melengestrol	>100	10	>100	>100
Metabolite B	5	>10	>10	>10
Metabolite C	10	>100	>100	>100
Metabolite D	10	>100	>100	>100
Metabolite E	0.1	10	>100	>100

The data show that MGA and its metabolites exert their biological action primarily as progestogens and secondarily as glucocorticoids. At relevant physiologic concentrations, no activity was demonstrated in either the AR or ERα assays.

Transcriptional activation of MMTV-Luc by full-length human PR was maximal in response to approximately 0.1 to 1 nM progesterone, 0.01 nM R5020, and 0.01 nM medroxyprogesterone acetate. These data indicate that all of the assays were performing correctly, exhibiting responses that were consistent with historical data and general biologic activity relationships. With MGA, 50-100% maximal transactivation of PR was observed at 0.01 nM but not at 0.005 nM. Metabolite E was the most active metabolite with activity (i.e. the minimum concentration resulting in 50-100% maximal transactivation) being reported at 0.1 nM but not at 0.01 nM. Metabolites B, C, and D were generally active at 1 to 10 nM, which represented much lower activity compared to MGA. Melengestrol began to exhibit activity at concentrations of 1-100 nM but response was <50-100% maximal at these concentrations.

Activity data resulting from MGA, its metabolites and melengestrol in the PR assay were analysed statistically using an analysis of variance, with the objective of determining the relative bioactivity of each compound compared to parent MGA. The results are shown in Table 5. The comparator compounds progesterone, R5020 and MPA, were not included in the statistical analysis.

Table 5 Relative activity of MGA metabolites versus MGA based upon the PR assay

Compound	Relative Activity (%)	95% Confidence Interval (%)
Metabolite B	0.16	0.03, 0.89
Metabolite C	0.23	0.05, 1.05
Metabolite D	0.09	0.02, 0.39
Metabolite E	8.59	1.88, 39.30
Melengestrol	0.85	0.02, 47.08

Large 95% confidence intervals were reported for Metabolite E and melengestrol (Table 5). With Metabolite E, this is attributed primarily to one of eight assay results where the 50-100% maximal response of the human PR occurred at 0.0005 nM MGA, in contrast to 0.01 nM for the other assays. The activity of Metabolite E in the PR assays was consistent at 0.1 nM with one isolated exception of 0.01-0.1 nM. When melengestrol was used as the test substance in PR assays, activity was reported to range from 0.001 nM to >100 nM. Such pronounced variability contributed to a large 95% confidence interval, which is not a concern since melengestrol demonstrated < 0.01% of the progestogen activity of MGA (Table 4).

The progestogenic activity of Metabolite E was further examined using a modelling approach. This approach differs from the statistical approach described above since it does not rely on discrete values, but instead utilises all data from the assays to interpolate activity between the discrete values. The induction level was determined for each experimental observation as the ratio of the observed optical unit response to the average baseline. These induction data were analysed using a mixed effects model analysis of variance from which the concentration least squares means for the compound were determined and converted to percentages of maximum MGA induction. For each compound, the percent of maximum induction values were then fitted to a logistic model. The predicted concentrations for 10%, 50% and 90% maximum induction for MGA and for Metabolite E were determined from the model. The ratio of Metabolite E to MGA was used to determine the relative biologic activity of Metabolite E at each induction level (10%, 50%, and 90%).

The least squares means with standard errors for MGA and Metabolite E are summarised in Table 6 and graphically illustrated in Figure 1.

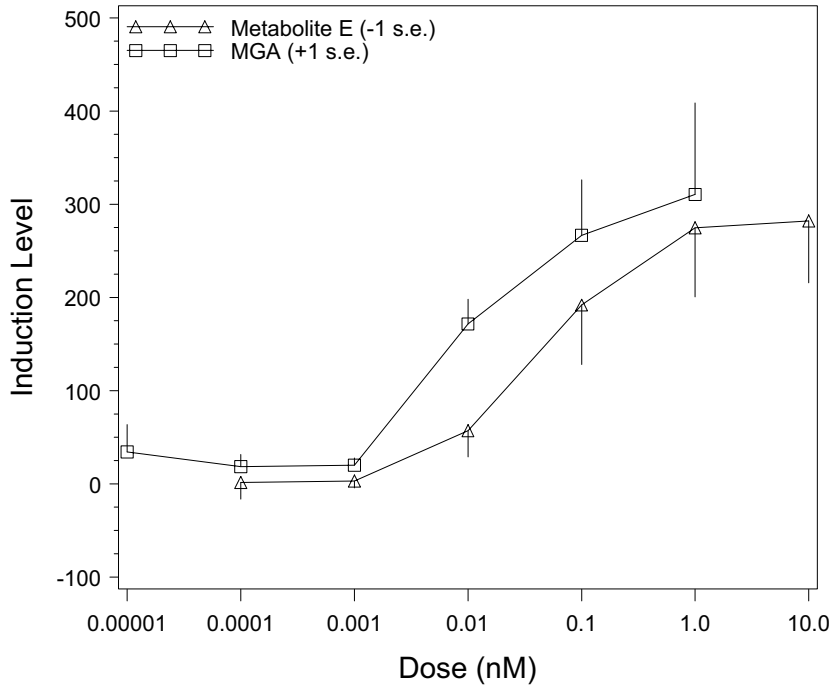
Table 6 Summary statistics for induction levels for MGA and Metabolite E

Concen-tration* (nM)	MGA				Metabolite E			
	n	LSMean	Std Err	Percent†	n	LSMean	Std Err	Percent†
0.00001	2	34.15	29.35	11.0	-	-	-	-
0.0001	6	18.43	12.86	5.9	3	1.49	18.04	0.5
0.001	7	19.99	7.60	6.4	7	3.04	7.60	1.0
0.01	8	171.57	26.33	55.3	7	57.00	28.15	18.4
0.1	8	266.65	59.62	85.9	7	191.79	63.74	61.8
1	4	310.45	98.12	100	7	274.57	74.19	88.4
10	-	-	-	-	4	282.12	66.43	90.9

* MGA was tested at 0.0005, 0.005, 0.05 and 10 nM in only one assay and because of lack of replication were not included in these analyses.

† Percent of the maximum MGA response in the LSMeans (these are the data used in the logistic models).

Figure 1 Plot of the least squares means +/- standard error for MGA and metabolite E (lines are simple line segments connecting each point)



The fit of the logistic models is shown in Figure 2 while the concentration to reach induction levels of 10%, 50% and 90% and the relative bioactivity (potency) of Metabolite E are shown in Table 7. The potency of Metabolite E relative to MGA was 12.2% at the 10% induction level, 12.0% at the 50% induction level, and 11.8% at the 90% induction level.

Figure 2 Plot of the logistic model fits and the LSMeans for MGA and Metabolite E.

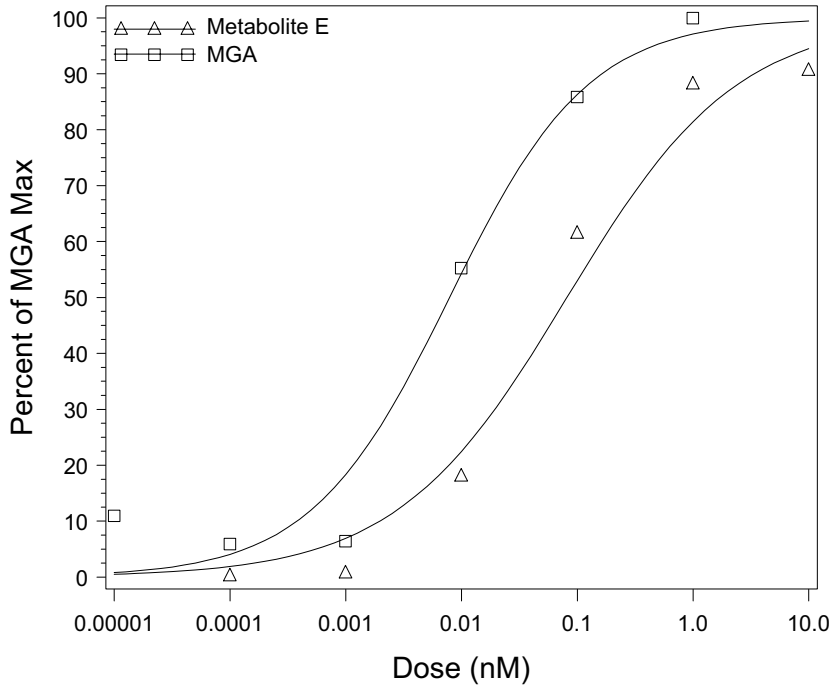


Table 7 The relative bioactivity of Metabolite E to MGA at various points on the curves (based on data from the logistic model).

% of Max MGA	Concentration of MGA* (nM/L)	Concentration of E* (nM/L)	Relative Activity of E to MGA
10	0.0005	0.0038	12.2%
50	0.0088	0.0733	12.0%
90	0.1677	1.4162	11.8%
*	As predicted by the models, the dose at which the % of maximum MGA is reached.		

APPRAISAL

Previous studies reported the extensive metabolism of MGA in several animal species and in humans. In vivo studies that investigated the fate of MGA have been conducted in cattle (Krzeminski et al, 1981), rabbits (Cooper et al, 1965), and women (Cooper et al, 1967). None of the metabolites of MGA formed in cattle were identified, whereas 6-hydroxymethyl-MGA and 2α-hydroxy-MGA were identified in the urine of rabbits. At least thirteen metabolites were generated in the urine of women, however, only one of these, 2α-hydroxy-MGA, was identified. In vitro studies into the metabolism of MGA have also been reported. Early experiments with bovine liver homogenates and rumen fluids were conducted using 3H-MGA (Janglan 1975a, 1975b). It was not possible to characterise the metabolites of MGA in these studies due to the limitations of analytical methodologies at the time. More recently, the extensive oxidative metabolism of MGA by hepatic microsomes prepared from rats, bovine, and human liver has been reported (Pfeiffer and Metzler, 2001). Although seven mono-oxygenated and five dioxygenated metabolites were observed in these studies, none of the metabolites was characterised further.

In the present studies, the metabolic profile of MGA was characterised following the generation and isolation of metabolites in in vitro test systems prepared from beef heifers. The metabolites were separated by HPLC and their structures characterised by HPLC, HPLC/MS and NMR. Three monohydroxy metabolites, one dihydroxy metabolite, and several trace metabolites were generated in bovine liver microsomes. Metabolites, from greatest to least abundance, were 2β-hydroxy-MGA (Metabolite E), 6-hydroxymethyl-MGA (Metabolite C), 15β-hydroxy-MGA (Metabolite D), and 2β,15β-dihydroxy-MGA (Metabolite B). The 2 β stereochemistry assigned to the hydroxyl moiety of Metabolite E differs from the 2α stereochemistry assigned arbitrarily by Cooper (1968), presumably reflecting the modern technology utilised in the present studies. Since Metabolite A was generated only in trace amounts, its structure could not be determined. Additional metabolites formed in trace amounts by bovine liver microsomal systems were identified as monohydroxy and dihydroxy products. Furthermore, no conjugation products or additional metabolites of MGA were observed in bovine liver slices or bovine liver S9 fractions.

Rat microsomes, human microsomes and human recombinant cytochromes P450 generated Metabolites B, C, D, and E, and additional minor metabolites. The latter were identified as monohydroxy and dihydroxy products. However, there were insufficient amounts for complete structure elucidation. Human P450 metabolism of MGA was shown to be primarily attributable to the CYP3A4 isoenzyme.

Using metabolites separated by semi-preparative HPLC in an in vitro cell receptor and gene expression system, the present studies concluded that MGA and its metabolites exert their biological action primarily as progestogens and secondarily as glucocorticoids. At relevant physiologic concentrations, no activity was demonstrated in either the androgen (AR) or oestrogen (ERα) receptor assays.

Since MGA and its metabolites were demonstrated to function primarily as progestogens, PR assay data were used to determine the relative bioactivity or potency (mg/kg dose resulting in equal pharmacological effect) of each compound compared to MGA. Metabolite E was shown to be the most potent of the metabolites when the pharmacodynamic data were analysed statistically using analysis of variance. The potency of Metabolite E relative to MGA, as measured by minimum induction concentrations that resulted in 50-100% maximal response, was estimated as 8.6% (i.e. 11.6-fold the dose of Metabolite E was required to achieve similar progestogenic activity as MGA). By comparison, the mean progestogen activities relative to MGA were 0.16% for Metabolite B, 0.23% for Metabolite C and 0.09% for Metabolite D.

The relative progestogenic activities of Metabolite E and MGA were subsequently compared by fitting concentration-effect curves using logistic modelling. In this analysis, all data from the assays were used to interpolate activity between the discrete test values. The concentration-effect curves for MGA and Metabolite E were parallel, indicating that both compounds act through the same receptor. The predicted concentrations of MGA and Metabolite E for 10%, 50% and 90% maximum response were determined. The potency of Metabolite E relative to MGA was 12.2% at the 10% induction level, 12.0% at the 50% induction level, and 11.8% at the 90% induction level.

The 54th meeting of the Committee noted that MGA, which is the marker residue, accounted for 85% of the total residues in fat and 33% of the total residues in liver (JECFA, 2000). Moreover, the ratio in total residues that was used to establish the MRLs for fat and liver was based upon radiolabelled metabolism studies in animals slaughtered under conditions consistent with zero-day withdrawal (6 hours after the last dose). The ratio of MGA residues in fat versus liver was 1.6:1. Based on the new information, the toxicological significance of the metabolites of MGA in tissue residues was considered further. Metabolite E, the most active metabolite, demonstrated on average 12% of the progestogenic potency of MGA i.e. on average requiring 8.8-fold the dose of MGA to achieve equipotent progestogenic activity. The relative potency of Metabolite E was then used to define the biological activity of the entire non-MGA fraction in the tissue residue, which potentially may be present in food for human consumption. This is conservative since the other metabolites (Metabolites B, C and D) had negligible activities ranging from 0.09% to 0.23% versus MGA. On the basis of the relative potency of Metabolite E, the non-MGA residues (fat 15%; liver 67%) were converted to MGA activity equivalents by reducing the percentage by a factor of 8.8. As shown in Table 8, 2.07% of the total progestogenic activity was attributable to non-MGA residues in fat and liver, respectively.

Table 8 Activity Weighting Factors for MGA-related residues in tissues

Tissue	% of total radioactive residue attributable to:		% of total progestogenic activity attributable to[b]:		
	MGA[a]	Non-MGA residues	MGA	Non-MGA residues	Sum of progestogenic residues
Fat	85	15	$\frac{85 \times 1 \times 100}{85 + (0.12 \times 15)}$	$\frac{15 \times 0.12 \times 100}{85 + (0.12 \times 15)}$	97.93 + 2.07 = 100
Liver	33	67	$\frac{33 \times 1 \times 100}{33 + (0.12 \times 67)}$	$\frac{67 \times 0.12 \times 100}{33 + (0.12 \times 67)}$	80.4 + 19.6 = 100
Kidney	<<LOQ	<<LOQ	-	-	-
Muscle	<<LOQ	<<LOQ	-	-	-

[a] Data from 54th JECFA

[b] The % of progestogenic activity of MGA-related residues is calculated by applying a weighting factor of 1 to MGA and of 0.12 (corresponding to the relative potency of Metabolite E) to all non-MGA metabolites, respectively, in fat and in liver.

The MRLs were subsequently derived by apportioning the ADI to the corrected total residues in fat and liver, in a ratio of 1.6:1 (Table 9). Accordingly, MRLs for cattle in fat and liver of 8 μg/kg and 5 μg/kg, respectively, were proposed.

Table 9 Theoretical maximum daily intake of MGA residues

Tissue	MRL (μg/kg)	Marker residue/ total residue[a]	Total residue (μg/kg)	Diet (kg)	Intake of residues ((μg)
Fat	8	0.979	8.2	0.05	0.41
Liver	5	0.804	6.2	0.1	0.62
TMDI					1.03

[a] This ratio is based on % of total progestogenic activity of the marker residue MGA as shown in column 6 in Table 8.

MAXIMUM RESIDUE LIMITS

In reaching its decision on MRLs for MGA, the Committee took the following factors into account:

- The established ADI is 0-0.03 μg/kg bw, which is the equivalent to up to 1.8 μg for a 60-kg person.
- The metabolites of MGA in in vitro test systems prepared from female cattle were identified as 2β,15β-dihydroxy-MGA (Metabolite B), 6-hydroxymethyl-MGA (Metabolite C), 15β-hydroxy-MGA (Metabolite D), and 2β-hydroxy-MGA (Metabolite E).
- Activation by MGA and its metabolites in in vitro test systems was most selective for the human progesterone receptor, which is consistent with historical in vivo data.
- Based on the submitted data, the biological activity of MGA-related residues in edible tissues of MGA-fed beef heifers can be principally attributed to MGA.
- The most active metabolite of MGA, 2β-hydroxy-MGA (Metabolite E), is 9-times less potent than MGA.
- A suitable regulatory method is available.

The Committee recommended MRLs in cattle of 8 μg/kg for fat and 5 μg/kg for liver, expressed as MGA. From these values, the theoretical daily intake of residues as MGA equivalents is 1.03 μg per person or 57.2% of the allowable ADI.

References

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PIRLIMYCIN

First draft prepared by

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IDENTITY

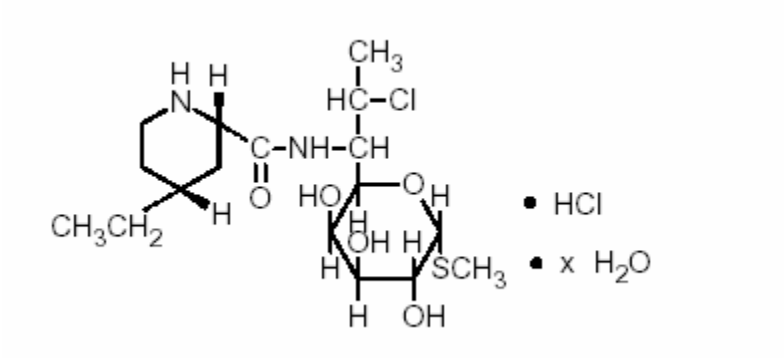
Chemical Names: (2S-*cis*)-Methyl 7-chloro-6,7,8-trideoxy-6-[[(4-ethyl-2-piperidinyl)carbonyl]amino]-1-thio-L-*threo*-alpha-D-galactooctopyranoside monohydrochloride, hydrate

Synonyms: Pirlimycin hydrochloride

PIRSUE® Sterile Solution

PNU-57930E

Structural formula:



Molecular formula: $C_{17}H_{31}O_5N_2ClS \bullet HCl \bullet xH_2O$

Molecular weight: 447.42 (without the water of hydration)

OTHER INFORMATION ON IDENTITY AND PROPERTIES

Pure active ingredient: Pirlimycin

Appearance: White crystalline powder

Melting point: 210.5 – 212.5°C with decomposition

Solubility (g/L) of Pirlimycin:	pH dependent aqueous:	70 at pH 4.5
		3 at pH 13
	Protic organic solvents:	≥ 100
	Other organic solvents:	≤ 10

Optical rotation: +170° to +190°

UV$_{max}$: >220 nm

RESIDUES IN FOOD AND THEIR EVALUATION

Conditions of use

General

Pirlimycin hydrochloride is a lincosamide antibiotic with activity against the Gram-positive organisms. Pirlimycin has been shown to be efficacious for the treatment of mastitis in lactating dairy cattle caused by sensitive organisms such as *Staphylococcus aureus, Streptococcus agalactiae, S. uberis* and *S. dysgalactiae*. The general mechanism of action of the lincosamides (lincomycin, clindamycin and pirlimycin) is inhibition of protein synthesis in the bacterial cell, specifically by binding to the 50s ribosomal subunit and inhibiting the peptidyl transferase, with subsequent interference with protein synthesis.

Dosage

The optimum dose rate for pirlimycin has been established as 50 mg of free base equivalents per quarter administered twice at a 24-hour interval by intramammary infusion of a sterile aqueous solution formulation. For extended therapy, daily treatment may be repeated for up to 8 consecutive days.

PHARMACOKINETICS AND METABOLISM

Pharmacokinetics in Laboratory Animals

Rats

Rats were treated with an oral gavage dose of 30 mg of ^{14}C-pirlimycin per kg of body weight as an aqueous formulation at 24-hour intervals for 5 consecutive days (Nappier, 1989). All animals were sacrificed at 2 to 4 hours after the last treatment. Approximately 88% of the administered dose was recovered in urine, feces and gastro-intestinal tract contents as shown in Table 1. There were no significant differences between male and female rats.

Table 1 Excretion of total ^{14}C-pirlimycin after dosing rats with 30 mg/kg/day for 5 days

	Percent of Total ^{14}C-Pirlimycin Dose	
Sample	Male rats	Female rats
Urine	4.5	6.4
Feces	62.8	58.8
Gastrointestinal Tract	20.6	22.5
Total	87.9	87.7

Mice

Pharmacokinetic studies were not conducted in mice.

Pharmacokinetics in Food Animals

General

The three studies have been conducted to examine the absorption, distribution, metabolism, and excretion of pirlimycin in the dairy cow following intramammary infusion of ^{14}C-pirlimycin (Hornish, 1988; Hornish, 1989a; Hornish, 1989b; Hornish, 1992a; Hornish, 1993c; Hornish, 1993d). Pirlimycin was readily labelled in the carboxyl carbon of the amide linkage and had a specific activity of 11.7 mCi/mmole (433 MBq/mmole) and a radiochemical purity of >98% (Hornish, 1988). The selection of this label site was based on the known metabolism of lincomycin and clindamycin (lincosamides structurally related to pirlimycin) in the dog, man, and rat (Daniels, 1976; Daniels, 1977; Eberts, 1967; Hornish, 1987; Onderdonk, 1981; Sun, 1973a; Sun, 1973b). These studies indicate that the lincosamides are not metabolized by cleavage of the amide linkage, which would expose the carbonyl carbon to subsequent metabolism and potential loss as carbon dioxide. Studies conducted in dairy cattle have demonstrated that this labelling site is metabolically stable for the complete delineation of the metabolism and residue fate of pirlimycin in the cow (Hornish, 1988).

Cattle

A GLP study was conducted in which 12 dairy cattle in mid-lactation were treated with ^{14}C-pirlimycin hydrochloride by intramammary infusion twice at a 24-hour interval at a dose of 200 mg/quarter (Hornish, 1988; Hornish, 1989a; Hornish, 1989b). This is four times the recommended dose. Blood samples were taken by jugular venipuncture at the times indicated in Table 2 and the total ^{14}C-pirlimycin free-base equivalents determined by combustion analysis of the whole blood (Hornish, 1988).

Table 2. Concentration of total ^{14}C-pirlimycin residues as a function of time in whole blood of dairy cows administered ^{14}C-pirlimycin by intramammary route at a dose of 200 mg/quarter twice at a 24-hour interval in each quarter

Sample Time Dose + Hour	Number of Data Points per Sample Time	Mean Concentration in Blood (µg/L)
D1 + 0.5	6	6
D1 + 1.0	12	11
D1 + 2.0	12	19
D1 + 4.0	12	38
D1 + 6.0	12	55
D1 + 8.0	6	53
D1 + 9.0	6	86
D1 + 10.0	6	64
D1 + 12.0	12	83
D1 + 16.0	12	54
D1 + 24.0	12	37
D2 + 6.0	12	119
D2 + 12.0	12	126
D2 + 24.0	12	63
D2 + 36.0	8	44
D2 + 48.0	12	38
D2 + 72.0	3	38

Concentrations in the blood were low but indicated that some of the drug was absorbed into the systemic circulation. The elimination phase suggested a bi-phasic pharmacokinetic model. Blood residues were not metabolically profiled. Analysis of the milk and urine samples collected during the terminal depletion phase showed that these samples contained >95% and >80% parent pirlimycin, respectively, suggesting that the blood residue was most likely composed of parent pirlimycin. Mean pharmacokinetic parameters were estimated following non-compartmental analysis (Hornish, 1988). Results are presented in Table 3.

Table 3 Whole blood pharmacokinetics of ^{14}C-pirlimycin total residue in the dairy cow following intramammary administration of 200 mg/quarter

Parameter	Value
AUC_{0-120}	2.27 to 7.11 µg-hr/mL
$t_{½}$ of abs. phase	2.89 ± 0.46 hours
C_{max-1}	0.083 ± 0.030 ppm
C_{max-2}	0.131 ± 0.047 ppm
K_{el}	0.0224 ± 0.009 hr^{-1}
$t_{½}$ of terminal phase	37.6 ± 17.4 hrs

The animals in the study were sacrificed at 4, 6, 14, and 28 days after last treatment (Hornish, 1988). Total milk at 12-hour intervals and urine and feces at 24-hour intervals were collected through 6 days after last treatment or until the animal was sacrificed. Total liver, kidney, udder, and samples of abdominal fat and flank and udder diaphragm muscle were harvested for total residue and metabolite determination. The results are presented in Table 4.

Table 4 Disposition and accountability of ^{14}C-pirlimycin total residue in the dairy cow following intramammary administration of 200 mg/quarter

Withdrawal Time (days)	Mean Percent of Total Administered Dose				
	Milk	Urine	Feces	Tissues[1]	Total
4	51.6	7.6	22.8	8.9	90.9
6	58.7	10.4	18.3	5.8	91.2
14	42.3	9.4	30.2	2.4	84.3
28	50.9	12.2	23.8	0.3	87.2
MEAN	50.9	9.9	23.8	4.4[2]	88.9

[1] Calculated from weight of whole liver, kidneys, udder and estimated muscle and fat weights as 55% and 25%, respectively, of total body weight at slaughter.

[2] Mean residue concentration over the withdrawal time range in tissues is for computation only and has no physiological significance.

Approximately 50% of the total dose was transported to the systemic circulation. Nearly 10% of the total dose was excreted via the urinary tract and 24% of the total dose was excreted via the GI tract through the 4 to 6 days of collection.

The depletion of total residue from the milk in the dairy cow studies was bi-phasic. A rapid initial phase was caused by unabsorbed pirlimycin being flushed from the udder during the first 3 or 4 milkings post-treatment (Hornish, 1988; Hornish, 1992a).

In a second GLP study (Hornish, 1992a; Hornish, 1993c), 23 cows were treated twice at a 24-hour interval in all four quarters with 50 mg ^{14}C-pirlimycin /quarter. The disposition of the total administered dose in milk (50.7%), urine (12.7%), feces (27.6%) and tissues (4.6%) gave an overall accountability of 95.7%.

In a third non-GLP study, three healthy lactating dairy cows (Hornish, 1993d) in mid-lactation were treated intravenously with a single infusion of 811 mg of ^{14}C-pirlimycin hydrochloride in sterile water. Blood samples were collected over a 7-day period. Following a four-week washout period, the cows received an intramammary infusion of 790 - 795 mg of ^{14}C-pirlimycin, approximately 200 mg in each quarter. Blood samples were again collected through 7 days. In addition, all milk, urine, and feces were collected for 7 days post-treatment following each dose. All samples were assayed for total radioactivity and for parent pirlimycin. The total residue results are summarized in Tables 5 and 6 (intravenous and intramammary administration, respectively) and the parent pirlimycin residue results are summarized in Table 7.

Table 5 Pharmacokinetics and disposition of total pirlimycin after intravenous (IV) administration of ^{14}C-pirlimycin to lactating dairy cows

Parameter	Cow 589	Cow 590	Cow 592
Model/Best Fit	Triexponential	Triexponential	Triexponential
A (ng/mL)	778.5±31.9	1547.5±145.4	794.1±124
Alpha (hr^{-1})	1.59±0.17	2.29±0.54	3.04±0.71
B (ng/mL)	293.2±46.7	270.9±200.6	342.0±132
Beta (hr^{-1})	0.06±0.02	0.09±0.12	0.54±0.24
C (ng/mL)	23.2±53.2	37.5±224.2	173.1±21.0
Gamma (hr^{-1})	0.004±0.019	0.01±0.07	0.018±0.004
$T_{½}\alpha$ (hours)	0.44	0.30	0.23
$T_{½}\beta$ (hours)	11.6	8.1	1.3
$T_{½}\gamma$ (hours)	173.3	70.0	38.5
AUC $_{0-\infty}$ ng*min/mL	10911.9	7642.5	10615.1

Table 6 Pharmacokinetics and disposition of total pirlimycin after intramammary (IMM) administration of ^{14}C-pirlimycin to lactating dairy cows

Parameter	Cow 589	Cow 590	Cow 592
Model/Best Fit	Triexponential[1]	Triexponential[1]	Biexponential[2]
A (ng/mL)	1771.8±63547	172.8±1440	
Ka (hr^{-1})	0.16±0.47	0.08±0.23	
B (ng/mL)	-1847.7±63551	-179.6±1457.5	110.3±66.2
Alpha (hr^{-1})	0.19±0.51	0.14±0.35	0.15±0.11
C (ng/mL)	49.0±20.8	27.4±31.7	623.5±231
Beta (hr^{-1})	0.01±0.005	0.005±0.009	0.01±0.006
$T_{½}$Ka (hours)	4.2	8.7	
$T_{½}\alpha$ (hours)	3.6	4.6	4.9
$T_{½}\beta$ (hours)	58.1	69.3	60.2
AUC $_{0-\infty}$ ng*min/mL	5157.2	6411.4	6072.9

[1] with 1st-order absorption

[2] with 0-order absorption

Table 7 Pharmacokinetics and disposition of parent pirlimycin after intravenous (IV) and intramammary (IMM) administration of ^{14}C-pirlimycin to lactating dairy cows

Parameter	IV	IMM
Dose (mg)	811	790-795
C_{max} (ng/mL)	N/A	62-96
T_{max} (hours)	N/A	9
$AUC_{0-\infty}$ (ng/mL/min)	3528-5510	1435-1868
Cl_B (mL/hr)	1.47-2.3 x 10^5	N/A
$T_{½}\alpha$ (hours)	0.16-0.27	10.5-12.6
$T_{½}\beta$ (hours)	10.8-23.1	
T_{abs} (0-order in hours)		7.2-7.9
MRT (hours)	17.9-33.7	
Vss (L)	4110-4960	
Excretion Recovery:	**IV**	**IMM**
% in milk	4.3 ± 0.7	40.2 ± 16.6
% in urine	26.5 ± 3.0	12.5 ± 2.6
% in feces	47.1 ± 1.7	29.7 ± 8.9
Total recovery	77.8 ± 2.2	82.5 ± 8.4

The bioavailability of pirlimycin in cattle following intramammary infusion was calculated to be 34% to 41%. The percent absorbed, measured as total ^{14}C-pirlimycin, residues was 51%.

Metabolism in Toxicological Test Species

Rats

The metabolism of pirlimycin was evaluated in the rat, the primary species used in the toxicological testing. Rats were treated by oral gavage once daily for 5 days with a dose of 30 mg of ^{14}C-pirlimycin per kg of body weight (Nappier, 1989) and sacrificed at 2 to 4 hours after the last treatment.

Liver was the tissue with the highest total residues and parent pirlimycin and the sulfoxide metabolite were the only residues found.

Mice

Metabolism studies were not conducted in mice.

Metabolism in Food Animals

Cattle

In the GLP study in which cows were treated by intramammary infusion twice at a 24-hour interval at a dose of 200 mg/quarter, milk samples were collected and analyzed by both an HPLC method and by a microbiological method (Hornish, 1989a). The results indicate that unchanged pirlimycin (by HPLC) comprised nearly 95% of the total residue in the milk, but the microbiologically active component in the milk was 106% of parent pirlimycin concentration measured by HPLC. Nearly all of the nonpirlimycin was found in the Dose + 12 hour samples and was attributed partially to unknown spurious spikes and partially to pirlimycin sulfoxide. These components contributed negligible amounts to total residue in other samples.

Residues in cattle liver were also examined by the two methods mentioned above (Hornish, 1989a). The HPLC analysis indicated that the residue consisted of only two components: pirlimycin sulfoxide as the major residue (76.5%) and unchanged pirlimycin as the minor residue (21.9%). The data demonstrate that the relative amounts varied over time, but are fairly constant in the critical 4-6 day withdrawal period as shown in Table 8. Parent pirlimycin is an acceptable residue marker since it is the only microbiologically active residue and is readily analyzed by a variety of methods.

Table 8 Percentage of total pirlimycin residue that is microbiologically active or parent pirlimycin in dairy cow liver following two intramammary infusions of 200 mg/quarter of ^{14}C-pirlimycin

Withdrawal Time (days)	Mean Percent of Pirlimycin in Liver by	
	M. luteus	HPLC/RAM
4	22.3	24.3
6	25.7	34.0
14	9.0	13.3
28	16.0	38.7

The metabolic profile of pirlimycin in the dairy cow for milk, liver, urine and feces (Hornish, 1989b) is summarized in Table 9.

The metabolism of pirlimycin was relatively simple. Pirlimycin sulfoxide was the only major metabolite isolated and was likely produced by oxidative hepatic processes. The sulfoxide, although the major residue in liver (65-75%), comprised only 5% of the excreted residue. The other pirlimycin residues identified in dairy cow liver were parent pirlimycin (22-25%) and pirlimycin sulfone (9.5%). Residues in the urine are about 80% pirlimycin and 8% sulfoxide; residues in the feces are about 45% parent and 2% sulfoxide. The remainder in urine and feces consists of adenylated adducts of pirlimycin and pirlimycin sulfoxide (Hornish, 1989b). Pirlimycin sulfoxide has approximately 1/100 (or 1%) of the microbiological activity of pirlimycin itself (Kennedy, 1991; Yancey, 1990; Yein, 1989a).

Table 9 Metabolic profile of the pirlimycin residues in the dairy cow following two intramammary infusions of 200 mg/quarter of ^{14}C-pirlimycin

Sample	Mean Percent Composition of Total Residue[1]		
	Pirlimycin	Sulfoxide	Other[2]
Milk	>95	<5	
Liver[3]	≈22	≈77	
Urine[4]	≈80	≈8	≈11
Feces[4]	≈45	≈2	≈50

1 Metabolite composition in each sample, not percent of total dose
2 Comprised of adenylated adducts of pirlimycin and pirlimycin sulfoxide
3 Average of 11 cows at 4, 6, 14, and 28 days withdrawal
4 Average of 12 cows through 4-6 days post last treatment.

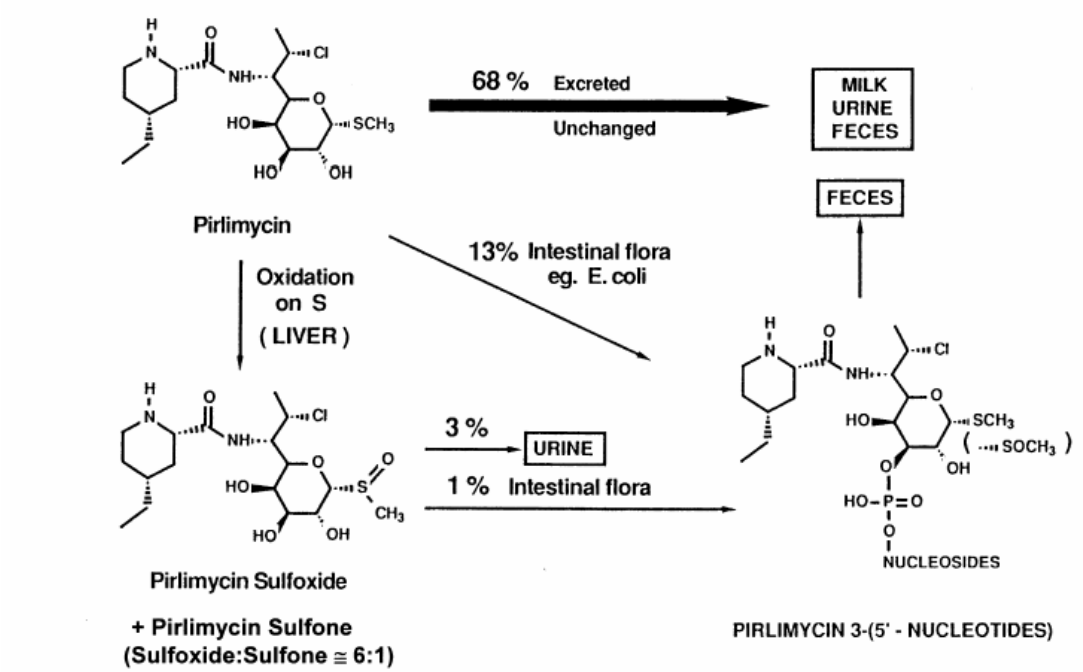
In cows dosed twice at a 24-hour interval in all four quarters with 50 mg ^{14}C-pirlimycin/quarter (Hornish, 1992a; Hornish, 1993c), four components were found in the liver metabolite profile. These were identified as pirlimycin (24.5%), pirlimycin sulfoxide (61.8%), and pirlimycin sulfone (9.8%).

Metabolites were identified in kidneys from four cows (three cows slaughtered 6 days after last treatment and one cow slaughtered 14 days after last treatment) (Hornish, 1993c). The mean composition of metabolites was 43.0% parent pirlimycin, 46.1% pirlimycin sulfoxide, and 7.2% pirlimycin sulfone. This composition is qualitatively similar to the composition in the liver. The concentration of total residue in the kidney was less than one-tenth the concentration in the liver at 10 days or more after last treatment.

Based on the studies described above, the metabolism of pirlimycin in the dairy cow resulting from the infusion of an aqueous solution of pirlimycin into the udder (intramammary route) is summarized in Figure 1 (Hornish, 1992b).

The various metabolites and residues of pirlimycin collected in milk, tissues, urine and feces all have significantly less microbiological activity (< 1%) than parent pirlimycin itself (Kennedy, 1991; Yancey, 1990; Yein, 1989a). Thus, parent pirlimycin is the key residue from a microbiological perspective and is an appropriate target analyte for residue monitoring purposes.

Figure 1 The metabolism scheme for pirlimycin in the dairy cow following intramammary administration of pirlimycin hydrochloride.



Based on the studies described above**,** liver is the tissue with the highest total residues of pirlimycin in rats and cattle. Parent pirlimycin and the sulfoxide were the only residues found, though the ratio of pirlimycin to pirlimycin sulfoxide was higher in the rat than in the cow. There was a good qualitative match of urine metabolites as well, but the two minor metabolites found in cow urine were not seen in the rat urine. Significant differences were observed in the fecal metabolite profiles, but those metabolites found in the cow feces that were not found in the rat feces have been postulated to arise from gut microflora deactivation and not from animal metabolism. These metabolites are not available to human consumers. The rat is considered a suitable species for toxicity testing of pirlimycin and its metabolites.

TISSUE RESIDUE DEPLETION STUDIES

Radiolabelled Residue Depletion Studies

Cattle

A GLP-compliant tissue residue depletion study (Hornish, 1992a; Hornish, 1993c) was conducted to determine the concentration of pirlimycin and total pirlimycin-related residues in the tissues of lactating dairy cows after treatment twice at a 24-hour interval with ^{14}C-pirlimycin in all four quarters at 50 mg/quarter. This is the recommended dose. A total of 23 cows were used in the study. Cows were slaughtered and tissues were harvested at 6, 10, 14, and 18 days after the last dose.

The disposition of the total administered dose in milk (50.7%), urine (12.7%), feces (27.6%) and tissues (4.6%) gave an overall accountability of 95.7%, as described above. The concentrations of total ^{14}C-residue found in the various tissues at the four slaughter time points are provided in Table 10.

Table 10 Mean residues of ^{14}C-pirlimycin in tissues of cows treated with 50 mg pirlimycin /quarter into all 4 quarters twice at a 24-hour interval

Post-treatment Interval, days (# cows)	Mean Concentration of Total ^{14}C-Pirlimycin Residue (µg/kg)*			
	Liver	Kidney	Muscle	Fat
6 d (n = 5)	2180 ± 1210	300 ± 210	18 ± 11	10 ± 10
10 d (n = 5)	1890 ± 1230	150 ± 80	11 ± 4	10 ± 10
14 d (n = 8)	990 ± 55	60 ± 40	7 ± 7	6 ± 2
18 d (n = 5)	890 ± 72	40 ± 30	< 5	< 5

*By combustion analysis and liquid scintillation counting.

Liver contains the highest residue at all time points. The concentration of total residue in the kidney was less than one-tenth the concentrations in the liver at 10 days or more after last treatment. Muscle and fat contain negligible concentrations of residue.

Residue Depletion in Milk

The same 23-cow GLP-compliant radiolabelled residue study was used to evaluate residues of pirlimycin in milk. The various milk samples collected throughout the study were analyzed for total ^{14}C-residues by scintillation counting procedures and for pirlimycin itself by the microbiological cylinder-plate analysis method (Yein, 1989b). The results of these analyses, Table 11, indicated that unchanged pirlimycin comprised >92% of the total residue "excreted" in milk by the microbiological cylinder-plate assay used in this study.

Table 11 Mean residues of ^{14}C-pirlimycin and Ratio of Parent Pirlimycin to Total Pirlimycin Residue in milk of cows treated with 50 mg pirlimycin /quarter into all 4 quarters twice at a 24-hour interval

Time (Hours) Post-treatment	Mean Pirlimycin Concentration, µg/kg		Ratio ‡
	Total residue *	*M. luteus* †	
Dose 1 + 12	19500	18000	0.91
Dose 1 + 24	2670	2470	0.90
Dose 2 + 12	18400	17000	0.93
Dose 2 + 24	2030	1770	0.89
Dose 2 + 36	420	380	0.90
Dose 2 + 48	170	150	0.93
Dose 2 + 60	110	100	0.96
Dose 2 + 72	80	70	0.95

* Concentration of total ^{14}C-residue determined by Liquid Scintillation Counting.

† Concentration of the microbiological activity (pirlimycin equivalents) based on the microbiological assay, not corrected for 95% recovery factor of the method.

‡ Based on the ratio of 23 samples per time point, not the ratio of the means.

Residue Depletion studies with unlabelled drug

Cattle - tissue residues

Three GLP-compliant studies were conducted to evaluate depletion of unlabelled pirlimycin in the tissues of cows.

In the first study, healthy cows were treated in either 2 (24 cows) or 4 (33 cows) quarters twice in a 24-hour period at a dose of 50 mg pirlimycin/quarter (Hornish, 1993a). The cows were slaughered at each of four time points (7 [4 quarter-treated only], 14, 21 and 30 days) after the last treatment. Liver residues were determined using the HPLC/TSP/MS without incubation and with the cylinder plate microbiological assay. The results are summarized in Table 12.

Table 12 Mean pirlimycin concentration (µg/kg) in cattle liver at each time point after 2 treatments with 50 mg pirlimycin in either 2 or 4 quarters

Withdrawal (days)	Treatment		
	2 quarters	4 quarters	Assay
7	- -	490±150 (430±110)	HPLC/TSP/MS (Cylinder plate)
14	90±40 (50±30)	70±30 (80±70)	HPLC/TSP/MS (Cylinder plate)
21	40±10 (30±10)	40±10 (60±30)	HPLC/TSP/MS (Cylinder plate)
30	50±10 (30±10)	60±30 (40±20)	HPLC/TSP/MS (Cylinder plate)

In the second study, four healthy cows were slaughtered at each of four time points (2, 7, 14, 21, and 28 days) after two treatments in all four quarters with 50 mg of pirlimycin (Hornish, 1997b). The results are summarized in Table 13. The table includes the results for “incubated” liver. This incubation step, which treats a subsample of liver at 37°C for 24 hours prior to the extraction step, was added to the sample preparation process when it was shown that the liver metabolite composition could change during sample preparation resulting in a reversion of pirlimycin sulfoxide to parent pirlimycin. This reversion was likely driven by residual enzyme activity left in the liver after necropsy (Hornish, 1998a; Hornish, 1998b; Hornish, 1998e).

Table 13. Mean pirlimycin concentration (µg/kg) * in 4 cows at each time point after 2 treatments with 50 mg pirlimycin in each quarter

Withdrawal (days)	Liver		Kidney	Muscle	Fat	Udder
	No Incubation	Incubation (24 hr at 37°C)				
2	1470±220	1690±210	460±70	20±30	<LOQ†	1040±350
7	240±40	610±190	60±10	<LOQ	<LOQ	150±120
14	<LOQ†	210±120	<LOQ	<LOQ	<LOQ	<LOQ
21	<LOQ	60±60	<LOQ	<LOQ	<LOQ	<LOQ
28	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

* HPLC/TSP/MS method
† LOQ = 25 µg/kg

In the third study, cows were treated for eight days (Hornish, 2000). In this study 5 cows were slaughtered at each post treatment time period. The results are presented in Table 14. Again, the table includes the results for "incubated" liver.

Table 14 Mean pirlimycin concentration (µg/kg)* in 5 cows at each time point after 8 treatments with 50 mg pirlimycin in each quarter

Withdrawal (days)	Liver		Kidney	Muscle	Fat	Udder
	No Incubation	Incubation (24 hr at 37°C)				
21	32±21	165±81	<LOQ†	<LOQ	<LOQ	<LOQ
28	21±22	165±182	<LOQ	<LOQ	<LOQ	<LOQ
35	28±18	96±67	NA‡	NA	NA	NA
42	<LOQ	42±46	NA	NA	NA	NA

* HPLC/TSP/MS method
† LOQ = 25 µg/kg
‡ Not assayed

In addition to the residue depletion studies conducted in healthy, non-mastic cows, an additional study was conducted in cows with an induced mastitis. These cows were then treated with four different regimens (Hornish, 1998d). Although these treatments were intended to evaluate the effectiveness of various extended-therapy regimens, animals were slaughtered and liver residue data were evaluated to assess whether the presence of mastitis affected the residue concentrations in the liver. All of the cows received a dose of 50 mg pirlimycin/quarter into all 4 quarters from one of the following treatment regimes: 2 doses at a 24-hour interval (8 cows); 5 doses at a 24--hour interval (8 cows); 8 doses at a 24-hour interval (8 cows); 6 doses with 36 hours between two consecutive daily doses at a 24-hour interval (8 cows). Samples of liver were assayed for pirlimycin residue using the HPLC/TSP/MS assay. These data are summarized in Table 15.

Table 15 Mean Pirlimycin Concentration (µg/kg)* in the Livers of Mastitic Cows at Each Time Point After various 50 mg Pirlimycin treatments in Each Quarter

Time after last treatment (days)	2 Doses	5 Doses	8 Doses	6 Doses
8		1000±230 (n=4)		1880 (n=1)
10	370 (n=1)			
15				750 (n=1)
16			50 (n=1)	
29	70±90 (n=7)	70±20 (n=4)	80±80 (n=7)	90±50 (n=6)

* HPLC/TSP/MS method (LOQ = 25 µg/kg)

The data for this study are insufficient to compute decline curves but suggest that residue depletion is similar in healthy and mastitic cows. Additionally, the data suggest that extended therapy does not greatly increase residues in the liver after several days of withdrawal.

Milk Residues

Three GLP-compliant studies were conducted to evaluate depletion of unlabelled pirlimycin in the milk of cows. In the first study, cows were treated in two quarters with pirlimycin at a dose of 50 mg/quarter twice in a 24-hour period (Hornish, 1993a). Milk residues were determined using the cylinder plate microbiological assay. Additionally, tissues were assayed using several screening tests. The results are summarized in Table 16.

Table 16 Mean residues (µg/kg) of pirlimycin in milk of cows following two daily intramammary doses of pirlimycin HCl at 50 mg/quarter into 2 quarters

Sample Time	Screening Test			
	Cylinder Plate (20 µg/kg)*	BSDA (70 µg/kg)	Delvotest-P (100 µg/kg)*	Charm II Macrolide (30 µg/kg)*
D2+12	4720±3050	32/32	32/32	32/32
D2+24	380±260	23/32	32/32	32/32
D2+36	100±50	7/32	6/32	32/32
D2+48	50±20	0/32	0/32	22/32
D2+60	30±10	0/32	0/32	12/32
D2+72	20±10	0/32	0/32	5/32

* estimated LOD of method

In the two- and eight-dose studies (Hornish, 1997a; Hornish, 2000) milk residue concentrations also were determined. Data from the two-dose study are summarized in Table 17 and residues from the eight-dose study are summarized in Table 18.

The concentrations determined using the cylinder plate assay and the HPLC/TSP/MS assay were nearly the same throughout the study, indicating that the pirlimycin residue measured using the HPLC/TSP/MS method corresponds to the microbiological residue measured with the bioassay.

Table 17 Mean residues of pirlimycin in milk of cows (n=20) following two daily intramammary doses of pirlimycin HCl at 50 mg/quarter into all 4 quarters

Sample Time	Pirlimycin concentration (µg/kg)	
	Cylinder Plate Assay*	HPLC/TSP/MS Assay**
Dose 1 + 12 hr	10300±4430	10300±4650
Dose 1 + 24 hr	820±1200	770±880
Dose 2 + 12 hr	13600±7180	10400±4990
Dose 2 + 24 hr	770±860	820±760
Dose 2 + 36 hr	220±230	210±310
Dose 2 + 48 hr	100±60	110±70
Dose 2 + 60 hr	50±20	70±20
Dose 2 + 72 hr	30±20	50±20
Dose 2 + 84 hr	30±10	(30±10)†
Dose 2 + 96 hr	20±10	(20±10)†

* LOQ = 20 µg/kg; LOD = 20 µg/kg ** LOQ = 50µg/kg; LOD = 20 µg/kg

† Values less than LOQ but greater than LOD

Table 18 Mean residues of pirlimycin in milk of cows (n=20) following eight daily intramammary doses of pirlimycin HCl at 50 mg/quarter into all 4 quarters

Sample Time	Pirlimycin concentration (µg/kg)*
Dose 8 + 12 hr	18600±12200
Dose 8 + 24 hr	1890±1800
Dose 8 + 36 hr	450±330
Dose 8 + 48 hr	160±40
Dose 8 + 60 hr	120±50
Dose 8 + 72 hr	80±30
Dose 8 + 84 hr	80±30
Dose 8 + 96 hr	50±20
Dose 8 + 108 hr	40±20

* Cylinder Plate Assay: LOQ = 20 µg/kg; LOD = 20 µg/kg

When the 2-dose and 8-dose treatments were compared, the depletion profiles for milk residues were not substantially different. The 2- and 8-dose treatments are compared in Table 19.

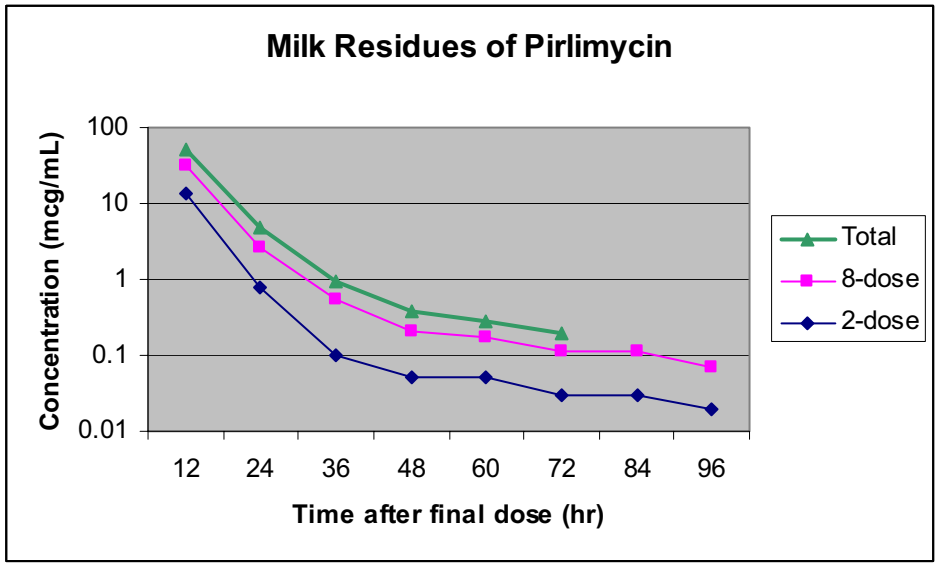
Table 19 Comparison of mean pirlimycin residues in the milk of cows following intramammary treatment at 50 mg/quarter into all 4 quarters for either 2 days or 8 days

Milk Sample	Pirlimycin concentration (µg/kg)*	
	2-Doses	8-Doses
12 hr after last treatment	13600±7180	18600±12200
24 hr after last treatment	770±860	1890±1800
36 hr after last treatment	220±230	450±330
48 hr after last treatment	100±60	160±40
60 hr after last treatment	50±20	120±50
72 hr after last treatment	30±20	80±30
84 hr after last treatment	30±10	80±30
96 hr after last treatment	20±10	50±20

* Cylinder Plate Assay: LOQ = 20 µg/kg; LOD = 20 µg/kg

Residues resulting from the 8-dose treatment were consistently higher (approximately 2X) than the residues resulting from the 2-dose treatment. This is shown graphically in Figure 2.

Figure 2: Mean concentrations of pirlimycin, determined using the cylinder plate bioassay, following 2-dose or 8-dose treatments at 50 mg pirlimycin/quarter into all 4 quarters and the total ^{14}C-pirlimycin residues from the radiolabelled depletion study.



Residues from the extended therapy study in mastitic cows are summarized in Table 20.

Table 20 Mean residues of pirlimycin in milk of mastitic cows (n=8) following various intramammary treatment regimes with pirlimycin HCl at 50 mg/quarter into all 4 quarters

Milk Sample	Pirlimycin concentration (µg/kg)			
	2 Doses	5 Doses	8 Doses	6 Doses
12 hr after last dose	6610±2340	7740±2080	6300±1710	5840±670
24 hr after last dose	420±90	990±420	650±340	450±100
36 hr after last dose	200±30	290±110	260±80	220±60
48 hr after last dose	100±20	120±40	120±30	100±30
60 hr after last dose	80±20	90±30	90±30	80±20
72 hr after last dose	60±20	70±10	70±30	70±40
84 hr after last dose	50±10	70±20	70±20	50±20
96 hr after last dose	40±10	50±10	40±20	40±20

The depletion profiles for milk residues were generally consistent, regardless of treatment regime. For the 2- and 8-dose treatments, residues in the milk of mastitic cows were generally lower than in the milk from healthy cows for milk collected through 36 hours after last dosing. Thereafter, the residues for mastitic cows and healthy cows were comparable. The results from the healthy and mastitic cows are compared in Table 21.

Table 21 A comparison of the mean residues of pirlimycin in the milk of healthy and mastitic cows after 2-dose or 8-dose treatment regimes with pirlimycin HCl at 50 mg/quarter into all 4 quarters

Milk Sample	Pirlimycin concentration (µg/kg)			
	Healthy Cows		Mastitic Cows	
	2 Doses	8 Doses	2 Doses	8 Doses
12 hr after last dose	13600±7180	18600±12200	6610±2340	6300±1710
24 hr after last dose	770±860	1890±1800	420±90	650±340
36 hr after last dose	220±230	450±330	200±30	260±80
48 hr after last dose	100±60	160±40	100±20	120±30
60 hr after last dose	50±20	120±50	80±20	90±30
72 hr after last dose	30±20	80±30	60±20	70±30
84 hr after last dose	30±10	80±30	50±10	70±20
96 hr after last dose	20±10	50±20	40±10	40±20

Three non-GLP residue studies were conducted to evaluate the effect of pirlimycin on starter cultures for cheeses, buttermilk/sour cream and yogurt (Hallberg, 1992; Hallberg, 1998a; Hallberg, 1998b). Pirlimycin concentrations tested were 140 to 590 µg/kg (Hallberg, 1992), 40 to 2400 µg/kg (Hallberg, 1998a) and 20 to 1280 µg/kg (Hallberg, 1998b). In all studies, the observed increase in clotting time was less than twice the clotting time for negative control milk. The lower 95% prediction value for average pirlimycin concentrations was 130 µg/kg. The study concluded that milk collected more than 36 hours after treatment would not adversely affect starter cultures. The study also noted that available milk screening assays could adequatedly detect pirlimycin and could be used to protect starter cultures.

METHODS OF ANALYSIS FOR RESIDUES IN TISSUES

Parent pirlimycin is the only significantly biologically active residue identified in milk and tissues and is, therefore, an appropriate marker residue for monitoring residues of pirlimycin in milk and tissues. Methods have been developed for the analysis of pirlimycin in both milk and tissues and are summarized in Table 22. There are two determinative methods for the quantitation of pirlimycin in milk and liver, one based on a microbiological assay (Benner, 1993; Yein, 1989b) and a second based on an instrumental HPLC/TSP/MS assay (Cazers, 1993; Hornish, 1991; Hornish, 1995a, 1995b). A third method for the specific identification and confirmation of parent pirlimycin in milk and liver is based on HPLC/TSP/MS (Hornish, 1995b). In addition, there are three commercially available screening assays for the detection of antibiotic residues in milk that have been tested against pirlimycin to establish the utility of these assays for detecting pirlimycin (Yein, 1992a; Yein, 1992b). These assays are the Delvotest®P (or Delvotest®SP), the *B. stearothemophilus* Disc Assay (BSDA), and the Charm II Test for Macrolide assay (Charm II) (Hornish, 1993a).

Milk

A highly specific mass spectrometric method is used for the simultaneous quantitative (determinative) and qualitative (confirmatory) determination of pirlimycin in milk. A thermospray interface is used to introduce the HPLC effluent into the mass spectrometer. Following chromatographic separation of sample components, a characteristic fragmentation pattern results in 4 principal ions which are detected by selective ion monitoring (protonated molecular pirlimycin, m/z 411). A stereoisomer of pirlimycin serves as an internal reference providing a marker for method recovery and HPLC retention time, and a normalizing ionisation control for the TSP response. A calibration curve is generated by varying the amount of pirlimycin while holding the amount of iso-pirlimycin constant and measuring the ratio of the peak area of the m/z 411 ion response for pirlimycin to iso-pirlimycin. Interference from endogenous matrix components is virtually eliminated by sequential extraction coupled with solid-phase extraction.

Milk samples are fortified with the internal standard, undergo an acidic extraction, are alkalinized and are cleaned up using a solid-phase extraction procedure (SPE). Following evaporation of the SPE product eluant, the final residue sample is re-dissolved for HPLC/TSP/MS analysis.

The method is validated over a range of 50-1200 µg/kg (Table 22, methods 2M and 3M). The method utilizes two concentration ranges (25 µg/kg to 200 µg/kg and from 200 µg/kg to 1200 µg/kg) that result in straight-line linear regression standard curves. The method has a recovery of 85-100% for determination and 100% for confirmation. The limits of quantitation are 50 and 100 µg/kg for the determinative and confirmatory assays, respectively.

The accuracy of the method was examined by analysing five sets of fortified control milk samples at four concentrations ranging from 0 to 800 µg/kg. These samples had been previously analysed by a validated *M. luteus* microbiological

determinative method (Table 22, method 1M). The quantitative analysis was based on the ratio of the peak area responses for pirlimycin to the internal standard for the principal pseudomolecular ion at m/z 411.4. The overall method recovery was 102%. The slope of the concentration added regressed on the concentration found was 1.031, with an intercept at 0.001, and a linear regression coefficient (R^2) of 0.9924.

The precision of the method was judged relative to the bioassay method (Hornish 1991). The day-to-day coefficient of variation (C.V.) of the determination of pirlimycin concentration in the range 200 to 800 µg/kg was ≤ 7%. The within day C.V. of pirlimycin recovery from the spiked samples was ≤ 6%.

The limit of detection (LOD) was estimated from the pirlimycin-free control milk samples in terms of the standard deviation ($SD_{\bar{x}}$ = 0.009) of the quantitative mean at the retention times of the analytes. The estimated LOD for this method based on the quantitative measurements of the m/z 411.4 ion at the appropriate retention times for pirlimycin and the internal standard is 40 µg/kg.

The estimated limit of quantitation (LOQ) was derived statistically where LOQ = quantitative mean + 10 SD. This resulted in an LOQ of 100 µg/kg. However, a subsequent study (Hornish 1995a) led to the revision of this figure down to a validated LOQ of 50 µg/kg, at which point the recovery was 85% and C.V. <8%.

Several parameters were examined to assess method ruggedness. Solid-phase extraction (SPE) column variability was tested by evaluating three lots of SPE columns using triplicate samples of control milk fortified to 400 µg/kg. There was no significant difference between lots. The effect of varying the organic concentration of the SPE elution solution was evaluated and no significant difference was detected (recoveries of 99.6±1.1% and 107.2±13.9%). Evaluation of different times to evaporate the SPE eluant showed that times greater than or equal to 30 minutes gave recoveries significantly different, and it was concluded that samples must not be left for more than 10-15 minutes after dryness is attained. Variability of HPLC columns was examined using different columns and different lots of column packing material. No significant differences were noted.

In the HPLC/MS system, the thermospray vaporiser performance represents the weakest part of the overall method. No performance deterioration (*i.e.*, as evidenced by increased backpressure and increased operating temperatures with subsequent loss of sensitivity and stability of the ion-flux.) attributable to deposition of non-volatile substances in or around the vaporizer orifice were encountered in the development of the HPLC/TSP/MS method.

The procedure typically takes 60 to 75 minutes for 6 samples to be processed to completion. As a result, the stability of the final solution to be analysed on the LC/MS system was evaluated. Several samples that had been analysed within hours of preparation, were re-analysed after 7 days storage at 2-4°C. The results showed that there was minimal loss of sample integrity within the bounds of the variability of the method (<10%). It was therefore concluded that prepared samples could be satisfactorily stored for several days at 4°C if necessary.

Tissues

Liver

The method for the simultaneous determination and confirmation of pirlimycin is based on the HPLC/TSP/MS method described for milk (Hornish, 1992c; Hornish, 1993b; Hornish, 1997c; Hornish, 1998a; Hornish, 1998b).

Liver samples are incubated at 37°C for 24 hours to maximize the reversion of pirlimycin sulfoxide back to parent pirlimycin. The sample is then fortified with the internal standard and undergoes an acidic extraction. The resulting slurry is filtered, the filter cake rinsed, and the combined filtrate partitioned to expel an aqueous phase containing the acid salt of pirlimycin. Additional recovery is obtained by extracting the organic solution with additional water. The combined aqueous solutions are partially evaporated, alkalinized, and further purified by extraction into methylene chloride. This extract is evaporated to dryness and the residue redissolved for HPLC/TSP/MS analysis. The method is validated over a concentration range of 25 to 2000 µg/kg (Table 22, methods 2LI and 3L).

The method was developed originally without the incubation step. Subsequently, it was demonstrated that pirlimycin concentrations increased in samples maintained at room temperature or at 37°C. The original method was validated over a concentration range of 100µg/kg to 1000 µg/kg. An amended method, now referred to as the low range method (Table 22, method 2LL), has been validated over a concentration range of 25µg/kg to 100 µg/kg (Hornish, 1993b). A high range method (Table 22, method 2HL) has been validated for a concentration of 500 to 10000 µg/kg.

Much of the validation work on the HPLC/TSP/MS assay was conducted before the phenomenon of increasing parent pirlimycin was defined and elucidated. The modified method, 2LI, differs from the previously validated methods only in the incubation of the kidney tissue before initiating the extraction/analysis procedure. Therefore, all performance criteria, except recovery and precision, remain the same.

As with the milk method, the HPLC/TSP/MS method is highly specific giving a characteristic fragmentation pattern, detecting 4 principal ions by Selective Ion Monitoring (SIM). Endogenous interference is virtually eliminated by the sequential extraction procedure.

Historically, the method is linear (correlation of (R^2) = 0.990) over the pirlimycin concentration range evaluated, 0 to 1000 µg/kg for 2LL and 2LI. Overall method recovery was 98% in the low and mid range assays, 0 to 500 µg/kg, and 500 to 1000

µg/kg (Hornish, 1997c), and 94% in the high range assay, 1000 to 5000 µg/kg (Hornish, 1997c). For the 2LI method, the mean recovery for incubated samples fortified at concentrations from 540 µg/kg to 2160 µg/kg was 76.4% (Hornish, 1998a).

Precision was evaluated for both the original and revised methods. In the original method, the day-to-day coefficient of variation (C.V.) of the determination of pirlimycin concentration in the range 100 µg/kg to 1000 µg/kg was 7.7%. The within day C.V. of the recovery of pirlimycin from the spiked samples was 5.2%. For the revised (2LI) method, the CV was 8.2% for fortified control samples, but was 12.4% for incurred-residue samples in the concentration range 240 µg/g to 1750 µg/kg (Hornish, 1998a).

The limit of detection (LOD) was estimated statistically (LOQ = mean + 3 SD) from the pirlimycin-free control liver samples. Consequently, the estimated LOD for this method based on the quantitative measurements of the m/z 411.4 ion at the appropriate retention times for pirlimycin and the internal standard is 40 µg/kg. However, during the validation, the operating LOD appeared to be 15 µg/kg (Hornish, 1998a).

The limit of quantitation (LOQ) was derived statistically (LOQ = mean + 10 SD), giving an estimated LOQ of 80 µg/kg (Hornish 1992c). A subsequent study (Hornish, 1998c) led to the revision of this figure down to a validated LOQ of 25 µg/kg, at which concentration the recovery was 85% with a C.V. <8%.

Several parameters were examined to assess the ruggedness of the method. No significant differences were noted based on degree of evaporation of the aqueous sample and there was no detrimental effect noted when the dried residue from the methylene chloride extraction was left for at least 15 minutes under flowing nitrogen and a water bath temperature of ≈70°C. Different HPLC columns and different lots of column packing materials were tested and no significant differences were found.

As noted with the milk method, the thermospray vaporizer performance represents the weakest part of the LC/MS method. No performance deterioration was encountered during the method development.

The extraction procedure (exclusive of the 24-hour incubation) typically takes 60 to 75 minutes for 6 samples to be processed to completion. Several samples that had been analysed within hours of preparation were re-analysed after 12 days storage at 2-4°C. The results showed that there was minimal loss of sample integrity within the bounds of the variability of the method (9.1%), where the ratio of the results at the two time points is not far from 1.0. It was therefore concluded that prepared liver samples could be satisfactorily stored for several days at 4°C if necessary.

Table 22 Analytical methods for the quantitative and confirmatory analysis of pirlimycin residue in milk and tissues

Matrix	Method ID	Method Description	Assay range	Recovery	LOQ	Ref.
Milk	1M	Quantitative Microbiological Cylinder Plate	20-320 µg/kg	95%	20 µg/kg	Yein, 1989b
Milk	2M	Quantitative HPLC/TSP/MS	50-1200 µg/kg	85-100%	50 µg/kg	Hornish, 1991; Hornish 1995a; Cazers, 1993
Milk	3M	Confirmatory HPLC/TSP/MS	≥100µg/kg	100%	100 µg/kg	Hornish, 1991; Hornish, 1995a; Cazers, 1993
Liver	1L	Quantitative Microbiological Cylinder Plate	40-160 µg/kg	78%	40 µg/kg	Yein, 1991
Liver	2LL	Quantitative HPLC/TSP/MS	25-1000 µg/kg	98%	25 µg/kg	Hornish, 1992c; Hornish, 1993b; Hornish, 1997c
Liver	2LH	Quantitative HPLC/TSP/MS	500-10000 µg/kg	94%	500 µg/kg	Hornish, 1997c
Liver	2LI	Quantitative HPLC/TSP/MS	250-2000 µg/kg	76%	250 µg/kg	Hornish, 1998a; Hornish, 1998b
Liver	3L	Confirmatory HPLC/TSP/MS	≥100 µg/kg	100%	100 µg/kg	Hornish, 1992c; Hornish, 1993b; Hornish, 1998b
Kidney	2K	Quantitative HPLC/TSP/MS	25-200 µg/kg	87-97%	50 µg/kg	Hornish, 1996; Hornish, 1998c
Muscle	2Mu	Quantitative HPLC/TSP/MS	25-200 µg/kg	86-97%	50 µg/kg	Hornish, 1996; Hornish, 1998c
Fat	2F	Quantitative HPLC/TSP/MS	25-200 µg/kg	90-100%	50 µg/kg	Roof, 1996; Hornish, 1998c

Kidney, Muscle and Fat:

This method for pirlimycin residue in kidney, muscle and fat also is based on the HPLC/TSP/MS method described for milk and liver (Hornish, 1996; Hornish, 1998b; Roof, 1996). These tissues do not require the incubation step necessary for liver because they contain parent pirlimycin as the principle residue. The tissue sample is fortified with the internal standard and undergoes an acidic extraction. Thereafter, the procedure is identical to the liver method. The operational range for the method is 25 to 2000 µg/kg (Table 22, methods 2K, 2Mu, and 2F).

Validations of the method for parent pirlimycin in kidney, muscle and fat were performed as above for the liver method. The quantitative assays for kidney (2K), muscle (2Mu) and fat (2F) all have an LOQ of 50 µg/kg and an LOD of 25 µg/kg. The confirmatory assay has a limit of confirmation (LOC) of 100 µg/kg.

APPRAISAL

Pirlimycin has not been previously reviewed by the Committee. Pirlimycin hydrochloride is a lincosamide antibiotic with activity against the Gram-positive organisms. It is used to treat mastitis in lactating dairy cattle. The drug is administered as an intramammary infusion at a dose of 50 mg pirlimycin/quarter.

Pirlimycin was found to be metabolized in a qualitatively similar manner in cattle and rats. Two minor metabolites were found in cow urine which were not identified in rat urine. Differences in the fecal metabolic profiles of cows and rats are attributable to gut microfloral deactivation and not animal metabolism. The rat appears to be a suitable species for toxicity testing for pirlimycin and its metabolites.

Radiolabelled residue studies were conducted in cattle at the labelled dose, 50 mg pirlimycin/quarter, and at an exaggerated dose, 200 mg pirlimycin/quarter. In all studies, all four quarters were treated. Residues in milk accounted for approximately half of the administered dose. Urine and feces accounted for approximately 13% and 28% of the administered dose, respectively. Residues in tissues were low, accounting for less than 5% of the administered dose.

Total residues in milk consisted almost entirely of parent drug. The concentration of parent drug in milk corresponds closely with the concentration of microbiologically active drug. Radiolabelled residues in milk deplete rapidly following the last dose.

In radiolabelled tissue residue depletion studies, total residues were highest in liver and were detectable for more than two weeks after dosing. Residues were readily detected in kidney but were approximately 10% of the concentration in liver. Significantly lower concentrations were found in muscle and fat. In liver, pirlimycin sulfoxide was the major residue and unchanged pirlimycin was the minor residue. The microbiological activity of parent pirlimycin is approximate 100 times that of the sulfoxide.

Parent pirlimycin is an appropriate marker residue as it represents the nearly all of the residues in milk and a significant, albeit minor, residue in liver. Pirlimycin also corresponds to the microbiologically active residues of concern.

In unlabelled residue studies, cows were treated at the labelled dose, 50 mg pirlimycin/quarter in all four quarters. In liver samples, an incubation step is added to the tissue extraction procedure to convert pirlimycin sulfoxide back to pirlimycin. Using the HPLC/TSP/MS method, residues are measured. Residues in muscle and fat are low or nondetectable at all sampling times (2 – 28 days after dosing). Residues are detected in kidney samples for the first week with means of 460 µg/kg and 60 µg/kg at 2 and 7 days respectively. Liver residues are present for an extended period of time, ranging from 1690 µg/kg at 2 days withdrawal to 60 µg/kg at 21 days withdrawal. In an extended therapy study, cows were treated for 8 days (*vs.* 2 days for the convention therapy) and liver residues persisted for 42 days withdrawal (mean residue = 42µg/kg at 42 days). In a study evaluating drug depletion in mastitic cows, a variety of treatment regimes were tested. In general, depletion profiles were similar for healthy and mastitic cows. Additionally, the extended therapy regimes did not result in significantly higher liver residues at later sampling times.

Milk residues also were evaluated using the 2-dose and 8-dose treatment regimes. Residues following the 8-dose treatment are consistently higher than the residues resulting from the 2-dose treatment at early time points. However, for samples collected more than 60-72 hours after the final treatments, these differences are small. In the mastitic cow milk residue study, residues of pirlimycin were lower than the residues in healthy cows for the first 36 hours after the last dose. Thereafter, milk residues were comparable for healthy and mastitic cows. After 48 hours, there was no significant difference in residue concentrations between the various treatment regimes.

Studies conducted to evaluate the effect of pirlimycin on starter cultures demonstrate that while clotting time is extended in milk containing pirlimycin, it is less than twice the time for negative control milk. Pirlimycin is unlikely to adversely affect the performance of starter cultures when a discard period of 36 hours or more is observed. Additionally, there are a number of screening tests available to detect pirlimycin in milk and protect starter cultures.

Parent pirlimycin is the only significant microbiologically active residue identified in milk and tissues. Methods are available to detect residues of pirlimycin quantitatively and qualitatively. In addition to a microbiological assay, a highly specific HPLC/TSP/MS method is available to measure residues of pirlimycin in tissues and milk.

For milk, the HPLC/TSP/MS method has a limit of quantification (LOQ) of 50 µg/kg and a limit of confirmation (LOC) of 100 µg/kg. The microbiological assay has an LOQ of 20 µg/kg. Recovery is generally good and the assay range is approximately 20-1200 µg/kg.

The HPLC/TSP/MS method can be used for the detection of residues in liver, kidney, muscle and fat. In liver, an incubation step is incorporated into the sample preparation. The range for quantitative analysis is 25-200 µg/kg (500-10000 µg/kg for the upper concentration range in liver). The LOQ is 50 µg/kg for kidney, muscle and fat and 25 µg/kg for liver (500 µg/kg for the upper concentration range in liver). As with milk, recoveries are good. In liver, the microbiological assay has an LOQ of 40 µg/kg and an assay range of 40-160 µg/kg.

The HPLC/TSP/MS method is suitable for monitoring residues of pirlimycin in milk and tissues but, as the method takes more than an hour to process 6 samples, it is considered only moderately practicable.

The Committee noted that the thermospray interface is no longer readily available. However, the method could be modified to use a currently available mass spectrometry interface.

MAXIMUM RESIDUE LIMITS (MRLS)

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

- An ADI of 0-8 µg/kg of body weight was established by the Committee based on a microbiological endpoint. This ADI is equivalent to up to 480 µg for a 60 kg person.
- Liver contains the highest concentration of total residues and is the target tissue for residue monitoring purposes. Pirlimycin is the principle microbiologically active residue in tissues and milk. In milk, pirlimycin accounts for nearly 95% of the total residues. Although pirlimycin sulfoxide represents a higher percentage (57-77%) of the total residues in liver than pirlimycin (22-25%), the microbiological activity of the sulfoxide is approximately 1% of pirlimycin. Therefore, pirlimycin is the marker residue in both tissue and milk.
- A validated HPLC/TSP/MS analytical method was use to measure residues of pirlimycin in milk and tissues in the studies submitted for the Committee's review and would be suitable for monitoring residues for regulatory purposes, but for the limitation noted above.
- Concentrations of pirlimycin below 130 µg/kg had no effect on bacterial starter cultures used in the production of fermented milk products.
- The MRLs recommended for liver and kidney were based on residue data from the unlabelled residue depletion study as determined with the HPLC/TSP/MS method. The MRLs recommended for muscle, fat, and milk are based on twice the LOQ for the analytical method.
- A statistical program developed for JECFA (Arnold, 2003) was used to facilitate the assignment of MRLs.

The Committee recommended permanent MRLs for pirlimycin in cattle of 1000 µg/kg in liver, 400 µg/kg in kidney, 100 µg/kg in muscle and fat, and 100 µg/kg in milk, determined as pirlimycin.

The MRLs recommended would result in a theoretical maximum daily intake of 305 µg or 64% of the ADI, based on the model daily food intake of 300 g muscle, 100 g liver, 50 g each of kidney and fat, and 1.5 kg of milk.

Tissue	MRL	Food Basket	TMDI
Muscle	100 µg/kg	0.3 kg	30 µg
Liver	1000 µg/kg	0.1 kg	100 µg
Kidney	400 µg/kg	0.05 kg	20 µg
Fat	100 µg/kg	0.05 kg	5 µg
Milk	100 µg/kg	1.5 kg	150 µg
TMDI			305 µg

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RACTOPAMINE HYDROCHLORIDE

First draft prepared by

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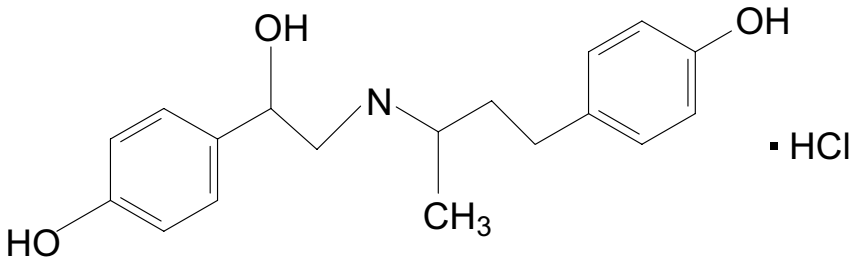
Dr. Stefan Soback, Beit Dagan, Israel

Supersedes the monograph prepared by the 40th Meeting of the Committee and published in FAO Food & Nutrition Paper 41/5

IDENTITY

Chemical name:	4-Hydroxy-α-[[[3-(4-hydroxyphenyl)-1-methylpropyl]amino]methyl] benzenemethanol hydrochloride {International Union of Pure and Applied Chemistry, or IUPAC, name}
	Benzenemethanol, 4-Hydroxy-α-[[[3-(4-hydroxyphenyl)-1-methylpropyl]amino]methyl]-hydrochloride {Chemical Abstracts Service (CAS) name; CAS number 90274-24-1}
Synonyms:	Ractopamine hydrochloride (common name); proprietary names: Paylean®, Optaflexx®

Structural formula:



Molecular formula:	$C_{18}H_{23}NO_3 \cdot HCl$
Molecular weight:	337.85 (HCl salt)

OTHER INFORMATION ON IDENTITY AND PROPERTIES

Pure active ingredient:	Ractopamine hydrochloride
Appearance:	Off-white to cream coloured solid
Melting point:	164-165.7 °C (USP Class I)
Solubility:	Soluble in polar solvents.
Stability:	Standards are stable at 20 – 25°C. Standard solutions should not be exposed to direct sunlight.
Ultraviolet maximum:	Maxima at 225 and 277 nm in methanol solution.

RESIDUES IN FOOD AND THEIR EVALUATION

In the sections which follow, concentrations are given as ractopamine hydrochloride equivalents, unless otherwise stated, based on the reports provided. Concentrations stated as ractopamine hydrochloride can be converted to equivalent free ractopamine by multiplying by a factor of 0.89 and using the same concentration units as stated in this report.

Conditions of use

Ractopamine hydrochloride is a phenethanolamine salt, which has been approved for use as a feed additive in some countries to enhance leanness in selected species. It is typically formulated by spraying an aqueous solution of the drug onto corn (maize) cob grits with the addition of 1-2% vegetable oil to reduce dust formation.

Dosage

The formulated product for swine, Paylean®, is recommended for continuous feeding to finishing pigs at concentrations of 5 - 20 mg/kg of feed to improve feed efficiency and increase rate of live weight gain for approximately the last 40 kg of body weight gain prior to slaughter, or at concentrations of 10-20 mg/kg in feed to increase carcass leanness and carcass dressing percent. The formulated product for cattle, Optaflexx®, is recommended for continuous feeding to finishing cattle at concentrations of 10 – 30 mg/kg feed for approximately the last 28 to 42 days prior to slaughter to increase the rate of weight gain, improve feed efficiency and increase carcass leanness.

PHARMACOKINETICS AND METABOLISM

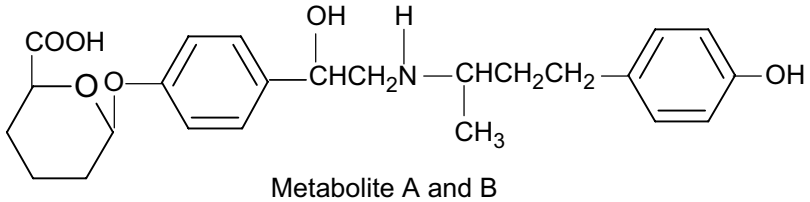
Laboratory Animals

Rats

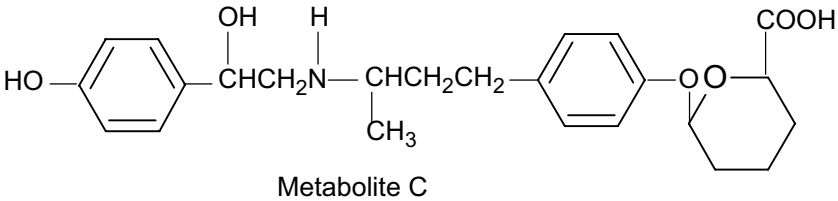
All studies in rats were performed using ^{14}C labeled ractopamine in which the radiolabel was attached in the hydroxyphenylethyl ring (ring "A") or the hydroxyphenylbutyl ring (ring "B") of ractopamine. The two differently radiolabeled ractopamines were then combined and used in radiochemically equivalent amounts.

Twenty-four Fisher rats (12 male, 12 female) received a daily dose of 2 mg/kg 14C-ractopamine hydrochloride (activity 0.489 μCi/mg) by gavage for 7 successive days (Dalidowicz, 1986a). Feces and urine were collected daily and pooled according to sex. The rats were killed six hours after the last dose and their livers and kidneys were collected and pooled according to sex. Three metabolites, designated as A, B and C, were separated using liquid chromatography and thin layer chromatography, then were characterized as monoglucuronides of ractopamine by fast atom bombardment mass spectrometry (FAB-MS) and nuclear magnetic resonance spectroscopy (NMR). In metabolites A and B, which are the RR,SS and RS,SR stereoisomers, the glucuronide is attached to ring "A" of the ractopamine structure. The glucuronide is attached to the "B" ring in Metabolite C, which was found to be a mixture of isomers. These three metabolites, shown in Figure 1, were stated to constitute a large portion of the 14C content in rat urine, but this was not quantified in the report of this study, which was conducted to GLP standards.

Figure 1 Structures of major metabolites, designated A,B and C, identified in urine and organs of rats.



Metabolite A and B



Metabolite C

In another GLP study, 24 Fisher rats (12 male, 12 female) received a daily dose of 2 mg/kg ^{14C}-ractopamine hydrochloride (activity 1.99 μCi/mg) by gavage for 5 days and were killed 3 hours after the last dose and livers and kidneys were collected (Dalidowicz, 1987a). Parent compound (ractopamine) was the major component (31.6%) of the extractable total radioactivity in the liver (0.40 μg/kg), but represented only 18.9% of the total radioactivity in kidney (0.33 μg/kg). Metabolites designated A, B, C, D, E, and F represented 12.0, 10.6, 7.0, 11.8, 0.3, and 6.7% of the remaining extractable radioactivity in the liver, respectively. In kidney, the metabolite distribution was 29.8, 32.8, 4.9, 5.6, 0.2, and 2.7%, respectively. The chemical structures of the metabolites were not further characterized in this study. Non-extractable residues were approximately 5.5% and 2.5% of the total radioactive residues in liver and kidney, respectively.

In a subsequent study, six rats, of which three had a bile-duct cannula and three others had both bile-duct and duodenal re-entry cannulas, were administered 2.85 ±0.30 mg (1.44 ±0.15 μCi) ^{14C}-ractopamine by gavage (Smith et al, 1995). Urine, faeces and bile were collected in three 8-hr. periods for the 24 hours following ractopamine administration, then the rats were killed. Absorption and excretion of the radioactivity was rapid, with 58 ±7% of the administered dose excreted in the bile during the

first 24 hours and 55% during the first 8 hours. Approximately 46% of the biliary radioactivity was identified as sulfate ester glucuronic acid diconjugate of ractopamine.

A bioequivalency study was conducted under GLP in which three groups of 10 F344/N Hsd BR rats (5 males, 5 females) received 0.5, 2.0, or 20 mg/kg 14C-ractopamine hydrochloride as a single oral gavage (Williams & al., 1985). The dose was equivalent to 3.8, 15.1 and 20 µCi/kg, respectively. Radioactivity was determined in plasma and whole blood from samples collected at 0.5, 2, 4, 6, 12 and 24 hours after dosing and calculated as µg ractopamine hydrochloride equivalents/mL. Absorption of the radiolabeled compound was rapid, as shown in Figures 2a (plasma) and 2b (whole blood).

Figure 2a **Concentration of ^{14}C-ractopamine hydrochloride (as µg-eq/mL) in plasma after single dose administration by oral gavage at 0.5, 2.0, and 20 mg/kg to rats.**

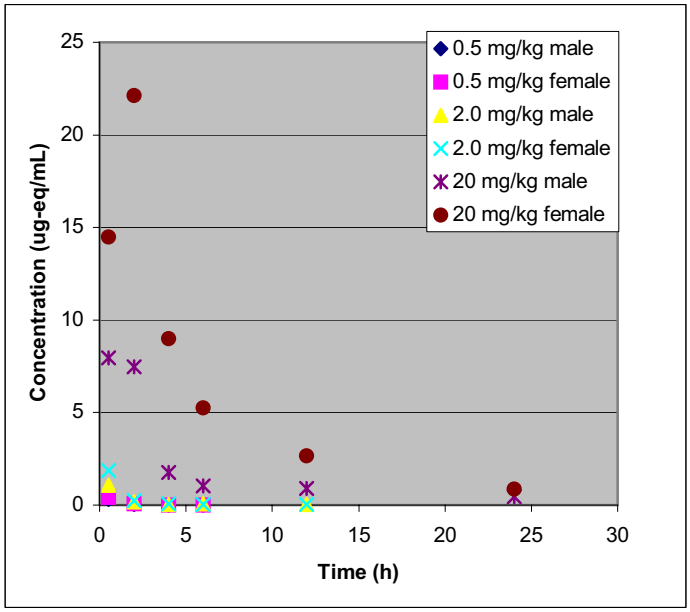
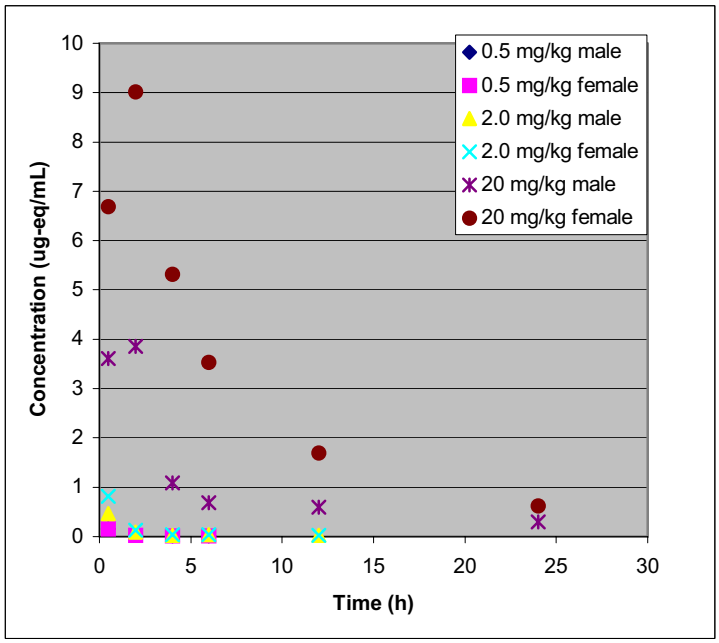


Figure 2b **Concentration of ^{14}C-ractopamine hydrochloride (as µg-eq/mL) in blood after single dose administration by oral gavage at 0.5, 2.0, and 20 mg/kg to rats.**



Peak drug concentrations in plasma and blood were proportional to the dose, appearing at 0.5 hours after administration, except for the highest doses, and were higher in female rats. The area under the concentration vs. time curve (AUC) increased proportionally to the increased dose at the two lower doses, but the increase was unproportionally large at the highest dose and was greater in female rats. This assessment, however, can not be considered accurate, because AUC was calculated for the total radioactivity related to a compound that undergoes considerable metabolism. The respective AUC was limited to the first 24

hours. Considerable part of the total AUC was not determined because extrapolation to time (24 hours) or to infinity was not attempted. Half-life could not be determined after administration of the lowest dose. The half life was 7.9 and 14.7 hours in the male rats after administration at 2.0 and 20 mg/kg, while in the female rats the respective half-lives were 5.0 and 7.0 hours. The results demonstrated that the majority of the measured radioactivity was associated with the plasma and not the whole blood under in vivo conditions.

Dogs

A GLP study was conducted in which two beagle dogs, a male and a female, were administered ^{14}C-ractopamine hydrochoride by gavage at 0.5 mg/kg three times daily for four days, with a single administration on the fifth day (Dalidowicz, 1986b). Urine and faeces were collected pre-administration and once daily until the dogs were sacrificed on the fifth day, 6 hours after the last dose, and livers and kidneys were taken for analysis. Analysis of urine by liquid chromatography and thin layer chromatography resulted in the separation and identification of the three metabolites designated A, B and C also identified in rat urine.

In a subsequent study, two beagle dogs, a male and a female, received 0.5 mg/kg of ^{14}C-ractopamine HCl three times daily by gavage for 4 days (Dalidowicz , 1987a). The animals were killed 3 hours after the last dose and their liver and kidney tissues were obtained for determination of compounds with ^{14}C-ractopamine-linked radioactivity. The same metabolites found in rat tissues were also present in dogs. Parent ractopamine accounted for 8.4% and 20.7% of the total extractable ractopamine related radioactivity in liver and kidney tissues. The respective concentrations were 0.59 and 0.50 mg/kg. The metabolites A, B, C, D, E, and F represented 6.4, 10.7, 23.9, 9.8, 3.4, and 3.8% of the remaining extractable radioactivity in the liver, respectively, and 7.4, 11.4, 25.4, 6.0, 6.0, and 10.8% in the kidneys. The major difference compared to the profile in rats was the proportionally lower concentration of metabolite A and higher concentration of metabolite C (see Figure 3a, 3 b).

Figure 3a Proportional composition of ractopamine and it's metabolites A, B, C, D, E, F in rat, dog, swine and cattle livers.

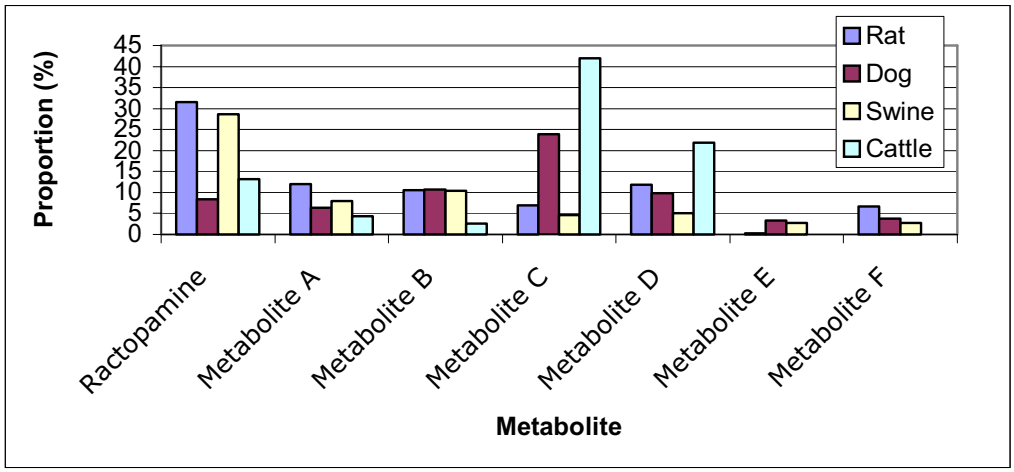
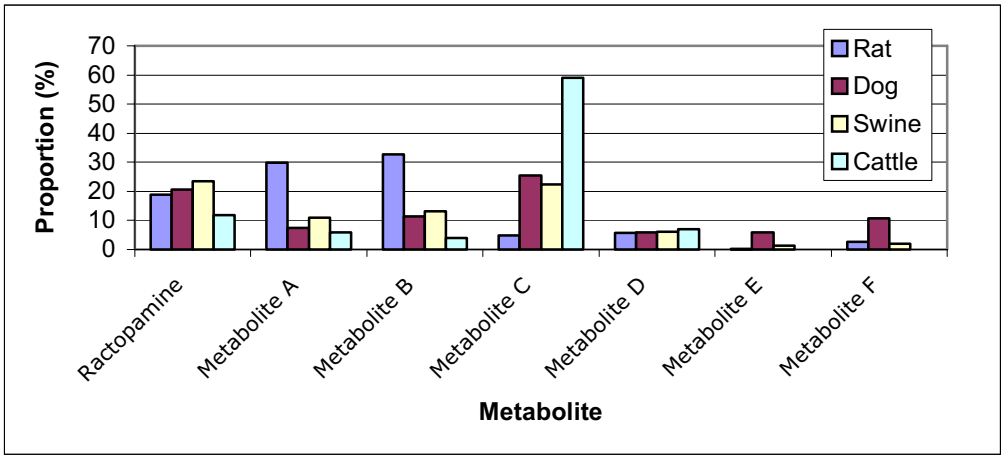


Figure 3b Poportional composition of ractopamine and it's metabolites A, B, C, D, E, F in rat, dog, swine and cattle kidneys.



Liver and kidney tissues from this study were also used in a GLP study on the comparative metabolism of ractopamine (Dalidowicz, 1990). The same metabolites described in the earlier studies were identified. Treatment of tissue extracts with β-glucuronidase Type IX (E. coli) released ractopamine, but treament with Type VI sulfatase (A. aerobacter) did not have an effect on the metabolites.

Monkey

A GLP study was reported, in which two rhesus monkeys were each administered 1.0 mL of 0.125 mg/mL ^{14}C-ractopamine hydrochoride (specific activity of 11.3 μCi/mg) by gavage (Williams, 1986). Urine and feces were collected at 24 hour intervals from 24 hours pre-treatment to 72 hours post-dose. Most of the radioactivity in urine was excreted during the first 24 hours. Altogether 69.8% of the radioactivity was collected in the excreta. Almost twice as much radioactivity was excreted in the urine compared to feces. The excretion pattern in monkeys was similar to that of the dog. A similar pattern of excretion has been observed in humans, where 45% of a single dose was excreted in the urine, mainly as sulfate conjugates, within 24 hours (Smith & Rodewald, 1994).

Food Producing Animals

Pigs

A GLP study was conducted in which three cross-bred pigs (each approx. 45 kg bw) were fed 1 kg feed containing 20 mg/kg unlabeled ractopamine twice daily for 5 days (Dalidowicz, Lewis & Thompson, 1986a). At the end of this period the animals received a single dose of 40 mg ^{14}C-ractopamine hydrochloride (0.5 μCi/mg) incorporated in the feed. After the administration of the radiolabelled compound, the pigs continued to receive feed twice daily containing unlabeled ractopamine for the duration of the experiment. The entire urinary and fecal output was collected from each animal at 24 hour intervals over a 7-day period, during which the animals excreted 96.5% of the ractopamine related radioactivity, of which 88.1% was via urine and 8.4% via feces. Of the total radioactive dose, 84.7% was excreted during the first day and 95.4% during the first three days. One pig was killed following day 7 due to illness, but sample collection continued for an additional 3 days from the two remaining pigs. There was no significant excretion of radioactivity in these samples.

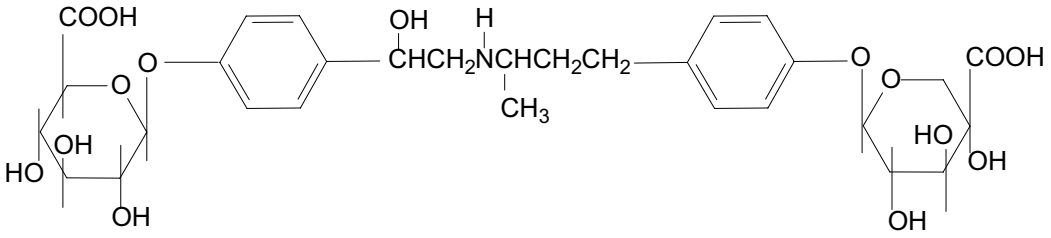
In another GLP study, 6 cross-bred pigs (each approx. 45 kg bw) received 30 mg/kg of ^{14}C-ractopamine hydrochloride in a special ration for 4 days, then killed 12 hours after the last dose (Dalidowicz, 1987b). Parent ractopamine accounted for 28.7% (0.12 mg/kg) and 23.4% (0.10 mg/kg) of the total extractable ractopamine-related radioactivity in liver and kidney tissues. Metabolites A, B, C, D, E, and F represented 7.9, 10.4, 4.6, 5.0, 2.7, and 2.8% of the remaining extractable radioactivity, respectively, in the liver, and 11.0, 13.2, 22.3, 6.1, 1.4, and 1.9% in the kidneys. While the pattern of metabolites was qualitatively equivalent to those observed in rats and dogs (see Figures 3a, 3b), the concentrations of ractopamine and the metabolites in the tissues in pigs were much lower.

Pigs were fed ^{14}C-ractopamine hydrochloride at the highest anticipated dose in a GLP study to determine the steady state concentration of the compound (Dalidowicz & al., 1984a) . Groups of three pigs (mixed male and female, each approx 50 kg. bw), received 30 mg/kg in feed for 4, 7 or 10 days. Each group was killed at 12 hours after their final feeding. Total concentrations of ^{14}C- ractopamine residues in muscle, kidney and liver tissue were 0.019-0.024, 0.466-0.655, and 0.254-0.424 mg/kg, respectively, for the administration periods. The steady state was reached in 4 days.

Cattle

Comparative metabolism of ^{14}C-ractopamine HCl was determined in a GLP study for cattle, dog and rats (Dalidowicz, 1990). The cattle tissues were obtained from two animals used in a previous residue study, details of which are given in a subsequent section of this report (Dalidowicz et al, 1987). Cattle metabolize ractopamine to four metabolites, A, B, C, and D (see Figures 3a, 3b). Metabolites A, B and C were previously characterized in the studies with rats and pigs. Metabolite C was the most abundant in cattle liver and kidney, at 0.25 mg/kg in each tissue. The fourth metabolite, D, was the second most abundant in cattle liver (0.13 mg/kg) and kidneys (0.03mg/kg), while metabolites A and B were at concentrations <0.03 mg/kg in both tissues. Experiments on bile extracts which included enzyme hydrolysis using β-glucuronidase Type IX (E. coli) and sulfatase Type VI (A. aerogenes) followed by liquid chromatography demonstrated that metabolite D was a glucuronide. Analysis by fast atom bombardment mass spectrometry revealed a major ion with m/z 653, which corresponds to the [M]+ , or molecular ion, for ractopamine diglucuronide. The probable structure of metabolite D is shown in Figure 4.

Figure 4 Sructure of ractopamine diglucuronide assigned to metabolite D isolated from bile of cattle.



Parent ractopamine accounted for 13.2% and 11.9% of the total extractable ractopamine related radioactivity in liver and kidney tissues. The respective concentrations were 0.08 and 0.05 mg/kg. Metabolites A, B, C, and D represented 4.3, 2.6, 42.0, and 21.8%, respectively, of the remaining extractable radioactivity in the liver, and 5.9, 3.9, 59.0, and 7.0% in the kidney. The

major difference compared to profiles in rat and dog was the proportionally higher concentration of metabolite C and lower concentrations of metabolites A and B. In the liver, the concentration of metabolite D was a significantly greater contributor to the total residue than in the other species studied.

A steady state GLP study in cattle was performed in which two cross-breed steers received 30 mg/kg non-radioactive ractopamine by gavage (Dalidowicz & Thomson 1989). After 8 days on a ration given twice daily containing 30 mg/kg unlabeled ractopamine hydrochloride, the cattle were given a single dose of ^{14}C-ractopamine hydrochloride at 0.67 mg/kg by gavage. Following treatment, the cattle continued on the diet containing 30 mg/kg unlabeled drug for 10 days, during which time the entire urinary and fecel output of the animals was collected daily. After 10 days the mean excretion of the ^{14}C-ractopamine was 97.8% and of this 45.6% was excreted in urine and 52.3% in feces, with 92.5% of the ^{14}C-ractopamine excreted in the four first days following the gavage treatment.

The urinary excretion of ractopamine and its metabolites was also reported in a separate study in which 6 heifers (315 ±21 kg) received a feed containing 20 mg/kg ractopamine hydrochloride (0.43 mg/kg bw/day) for 8 days, supplemented by hay ad libitum (Smith & Shelver, 2002). Urine was collected once daily from each animal, beginning on the day prior to introduction of the medicated ration and continuing for 7 days after change to a ration containing no ractopamine hydrochoride. Ractopamine was excreted primarily as conjugates. The mean concentration of parent compound in urine at the start of the withdrawal was 164 ± 62 µg/L. After hydrolysis, mean ractopamine concentration in these samples was 4129 ± 2351 µg/L. Additional details are provided in the following section of the report dealing with residue studies.

TISSUE RESIDUE DEPLETION STUDIES

Radiolabeled Residue Depletion Studies

Pigs

Six pigs (3 male, 3 female), each approximately with 50 kg bodyweight, received feed containing ^{14}C-ractopamine hydrochloride (30 mg/kg) for 7 days, after which one male and one female were slaughtered at 6 hr, 3 days and 5 days subsequent to final administration (Dalidowicz et al, 1984b). Liver, kidney, muscle and fat were tested for total radioactive residues using scintillation counting. One animal, which served as a control, received non-medicated feed and was also slaughtered with the group at 6 hr. Total residues were highest in kidneys at 6 hr (0.74 mg/kg), declining to 0.02 mg/kg at days 3 and 5 post-administration. Residues in liver were 0.18 mg/kg at 6 hr, declining to 0.09 mg/kg at day 3 and 0.04 mg/kg at day 5. Muscle and fat contained 0.03 and 0.02 mg/kg of residues, respectively, at 6 hr, with only traces detectable in samples on the other sampling days. A statement of GLP compliance was included in this report.

In a subsequent GLP study, another 6 pigs (3 male, 3 female; average bodyweight approximately 45 kg) also received a ration containing 30 mg/kg ^{14}C-ractopamine hydrochloride for four days, after which two pigs (1 male, 1 female) were slaughtered at each of 12 hr, 1 and 2 days post-administration (Dalidowicz, Thompson & Herberg, 1986). Total radioactive residues were determined in kidney, liver, muscle and fat. As in the previous study, an untreated animal served as control. Highest initial residues (0.46 mg/kg) were in kidney at 12 hr, declining to 0.13 at day 1 and 0.06 at day 2 post-administration. Residues in liver were 0.31 mg/kg at 12 hr, 0.17 mg/kg on day 1 and 0.07 mg/kg on day 2, while residues in muscle and fat were 0.01 mg/kg at 12 hr and 1 day and not quantifiable on day 2. Liver and kidney samples at 12 hr contained 0.08 and 0.02 mg/kg, respectively, of non-extractable residues, declining to 0.04 and 0.01 mg/kg on day 2 post-administration.

Another GLP study was reported in which 12 pigs (6 male, 6 female; each approximately 50 kg bodyweight) received a ration containing 30 mg/kg ^{14}C-ractopamine hydrochloride for four days, then were slaughtered in groups of 3 at 12 hr, 2, 4 and 7 days post-administration (Dalidowicz et al, 1985a). An untreated animal was used to provide control tissues. Total residues in kidney were 0.60 mg/kg at 12 hr, 0.06 mg/kg on day 2, 0.03 mg/kg on day 4 and 0.02 mg/kg on day 7 post-administration. In liver, the residues found at the same times were 0.42, 0.10, 0.05 and 0.06 mg/kg, respectively. Residues were found at the limit of detection (0.02 mg/kg) in muscle and fat at 12 hr and were not detected in the subsequent samples. Non-extractable residues in kidney accounted for 0.08 mg/kg of the residues at 12 hr and were not detectable in samples for the other days, while non-extractable residues in liver were 0.12 mg/kg at 12 hr, 0.06 mg/kg on day 2, 0.04 mg/kg on days 4 and 7 after final administration (Dalidowicz, 1987b).

Six pigs (3 male, 3 female, each approx. 45 kg bodyweight) that were fed a ration containing 30 mg/kg ^{14}C-ractopamine hydrochloride for four days were slaughtered at approximately 12 hours after the last feeding (Dalidowicz, Lewis & Thompson, 1986b). Total ^{14}C –containing residues were determined by combustion and scintillation counting, while residues of parent compound were determined by liquid chromatography with electrochemical detection. The total residues in kidneys averaged 0.41 ±0.04 mg/kg, of which 23.4% was parent compound. Total residues in liver were 0.41 ±0.06 mg/kg, of which 27.2% was parent compound. Based on these results, the ratio of total-to-marker residues in pig livers and kidneys at slaughter 12 hours following the last dietary exposure, or at effective "zero withdrawal" is approximately 4:1. A statement of GLP compliance was included in the report.

A subsequent study was reported in which two experiments were conducted using a total of 16 pigs (8 male, 8 female; each approximately 50 kg bodyweight), with the pigs receiving a ration containing 20 mg/kg ^{14}C-ractopamine hydrochloride for seven days (Dalidowicz et al, 1991). This study was conducted in compliance with appropriate FDA and OECD GLP guidelines, with the exception of a few minor deviations that were not considered by the Quality Assurance Unit which reviewed the work to have affected the study results. In the initial experiment in which 4 pigs received the medicated feed, two pigs (one male, one female) were slaughtered at each of 24 and 48 hours after the last administration of medicated feed. In

the subsequent experiment, in which 12 pigs received the medicated feed, 4 pigs (2 male, 2 female) were slaughtered at each of 24, 48 and 72 hours after final treatment. Tissues were analyzed by combustion and scintillation counting for total ^{14}C-residues and by liquid chromatography with electrochemical detection for parent drug. The residues of total and marker residue found in livers and kidneys in the experiments are summarized in Table 1. Based on these results, the ratios of total-to- marker residues in pig liver are 7:1, 20:1 and 33:1 at 24, 48 hours and 72 hours withdrawal, respectively. In kidney, the ratios for the same periods (total-to-marker) are approximately 4:1, 6:1 and 10:1.

Table 1 Total ^{14}C-residues and residues of parent drug found in livers and kidneys of pigs which received feed containing 20 mg/kg ^{14}C-ractopamine hydrochloride for seven days.

Time from last racto-amine exposure (hours)	n	Residues of ractopamine in liver (mg/kg)			Residues of ractopamine in kidney (mg/kg)		
		Total ^{14}C Residue	Parent Drug	Ratio Total : Parent	Total ^{14}C Residue	Parent Drug	Ratio Total : Parent
24	6	0.106 ±0.030	0.015 ±0.007	7 : 1	0.116 ±0.014	0.032 ±0.015	3.6 : 1
48	6	0.073 ±0.028	0.004 ±0.002	18 : 1	0.048 ±0.007	0.008 ±0.002	6 : 1
72	4	0.056 ±0.010	0.002 ±0.001	28 : 1	0.036 ±0.001	0.003 ±0.002	12 : 1

Cattle

In an initial GLP study, 6 steers (144-163 kg bodyweight) received a capsule containing 1.25 mg/kg bw of ^{14}C-ractopamine hydrochloride twice daily for seven days (Dalidowicz et al, 1985b). Two steers were slaughtered at each of 12 hr, 4 and 7 days after final treatment and an untreated steer was slaughtered to provide control tissues. The results from one steer slaughtered at 12 hr were excluded as this animal had received treatment for laryngo-pharyngitis during the trial period and this may have affected the residue depletion results. Five replicate test portions of liver, kidney, muscle and fat from each animal were analyzed for total radioactivity by scintillation counting. Highest residues were in liver (1.27 mg/kg) and kidney (0.97 mg/kg) at 12 hours after final treatment, declining to 0.17 mg/kg in liver and 0.19 mg/kg in kidney at day 4 and 0.09 mg/kg in liver and 0.11 mg/kg in kidney at day 7. Residues in muscle were 0.04 mg/kg at 12 hr, 0.02 mg/kg at day 4 and not detectable at day 7, while fat contained 0.05 mg/kg at 12 hr and no detectable residues in subsequent samples. The detection limit for ractopamine was approximately 0.02 mg/kg in all four tissues in this study.

In a subsequent GLP-compliant study, 6 steers and 6 heifers (177-236 kg bw) received 1.12 mg/kg bw per day for seven days of ^{14}C-ractopamine hydrochloride in a gelatin capsule by rumen insertion, a dose equivalent to 45 mg/kg in feed (Dalidowicz et al, 1987). A seventh steer served as an untreated control. Three animals (mixture of steers and heifers) were slaughtered at 12 hr, 2, 4 and 7 days after final administration of ractopamine. Total radioactivity was determined in liver, kidney, muscle and fat samples from each animal by liquid scintillation counting, while residues of parent compound were determined by liquid chromatography with electrochemical detection. Results of the analyses of livers and kidneys are given in Table 2. At 12 hr, total residues in muscle and fat were 0.02 . 0.00 and 0.01 . 0.00 mg/kg, respectively, with no detectable parent compound. No total or parent compound residues were detected in muscle and fat from the subsequent sampling dates.

Table 2 Total ^{14}C-residues and residues of parent drug found in tissues of cattle which received 1.12 mg/kg bw ^{14}C-ractopamine hydrochloride by rumen insertion on seven successive days.

Withdrawal Time (days)	Residues of ractopamine in tissues (mg/kg)					
	Liver			Kidney		
	Total	Parent	Ratio, Total : Parent	Total	Parent	Ratio, Total : Parent
0	0.62 ±0.13	0.14 ±0.04	4.4 : 1	0.46 ±0.07	0.06 ±0.01	7.7 : 1
2	0.08 ±0.03	0.02 ±0.00	4 : 1	0.10 ±0.03	0.01 ±0.00	10 : 1
4	0.06 ±0.02	-[a]		0.07 ±0.02	---a	
7	0.03 ±0.00	-[a]		0.04 ±0.01	---a	

[a] Not analyzed.

A GLP study was conducted in which 3 cattle (1 steer, 2 heifers, 166-230 kg bw) received ^{14}C-ractopamine hydrochloride for 7 days by intra-rumenal insertion of a gelatin capsule containing 0.67 mg/kg/day, a dose equivalent to 30 mg/kg administered in feed (Dalidowicz and Thompson, 1989b). The animals were killed approximately 12 hours after the final treatment and livers and kidneys were collected for analysis. Untreated control materials were obtained from a previous experiment. Total ^{14}C-residues were determined by liquid scintillation counting, while residues of parent compound were determined by liquid chromatography with electrochemical detection. Total residues found in liver were 0.25 ±0.10 mg/kg, of which parent

compound comprised 0.04 ±0.03 mg/kg. Total residues in kidneys were 0.19 ±0.02 mg/kg, 0.04 ±0.01 mg/kg of which was parent compound. The ratio of total residue to marker residue at 12 hr, considered as a practical "zero withdrawal" period, was approximately 6:1 for liver and 5:1 for kidney.

A larger GLP study was conducted in which 12 cattle (6 heifers, 6 steers; 183-231 kg bw) received ^{14}C-ractopamine hydrochloride for 7 days twice daily by insertion of a gelatin capsule via a rumen fistula (Smith & Moran, 1995). Each capsule contained a dose equivalent to 40 mg/kg (0.90 mg/kg bw/day/animal) administered in feed. The animals were kept in metabolism cages during the experiment and slaughtered in groups of 4 (2 heifers, 2 steers) at 48, 96 and 144 hours following the last treatment. Samples of liver, kidney, loin muscle and abdominal fat were collected from each animal and total ^{14}C-residues were determined by liquid scintillation counting. Residues of parent compound were determined using liquid chromatography with fluorescence detection (LOQ, 0.001 mg/kg for all tissues), but were not corrected for recovery. An untreated steer served as a source of control tissue. Based on the results in Table 3, the ratios of total residues to parent drug in liver and kidney tissues, are 39:1 and 23:1 (48 hrs), 70:1 and 25:1 (96 hrs), 54:1 and 89:1 (144 hrs), respectively. Analysis of retina from the treated animals revealed no detectable ^{14}C-residues in any of the samples. Differences in residue distribution obtained in this study when compared with other studies reported were not explained.

Table 3 Total ^{14}C-residues and residues of marker residue) parent drug in tissues of cattle which received ^{14}C-ractopamine hydrochloride via rumen fistula for seven days at a dose corresponding to 40 mg/kg in feed.

Time after final treatment (hours)	n	Residues in tissues (mg/kg)			
		Liver		Kidney	
		Total	Parent	Total	Parent
48	4	0.156 ±0.081	0.004 ±0.002	0.239 ±0.077	0.010 ±0.004
96	4	0.140 ±0.089	0.002 ±0.0011	0.148 ±0.058	0.006 ±0.005
144	4	0.054 ±0.010	0.001 ±0.0011	0.089 ±0.016	0.001 ±0.000[1]

[1] A value of 0.0005 mg/kg, equal to one-half the Limit of Quantification of 0.001 mg/kg, was assigned for results indicating detectable, but below the LOQ concentrations, in calculating the mean. The Limit of Detection was 0.0003 mg/kg.

Residue depletion studies with unlabeled drug

Pigs

In an initial GLP study, 12 pigs (6 male, 6 female) received a ration containing 30 mg/kg ractopamine hydrochloride for 7 days and were killed in groups of 6 (3 male, 3 female) at 12 and 24 hours after the final exposure to medicated feed (Lewis et al., 1987). A third group of 6 pigs (3 male, 3 female) received non-treated feed and served as controls. The bodyweight of the animals ranged from 74-91 kg pre-treatment and 83-103 kg at slaughter. Livers and kidneys were collected at slaughter and analyzed for ractopamine by liquid chromatography with electrochemical detection. No residues were detected in the tissues from the controls. In the animals killed 12 hours after final exposure, residues of parent compound in livers and kidneys were 0.058 ±0.027 mg/kg and 0.118 ±0.054 mg/kg, respectively. At 24 hours after cessation of ractopamine administration, the residues of parent compound in livers and kidneys were 0.022 ±0.010 and 0.031 ±0.016 mg/kg, respectively.

In a subsequent GLP study, pigs received a ration containing either 10 or 15 mg/kg ractopamine for 6 days (Turberg et al, 1991a). Three pigs (1 male, 2 female) received the ration containing 10 mg/kg ractopamine hydrochloride, while 13 pigs (7 male, 6 female) received the ration containing 15 mg/kg of the drug. Two pigs (1 male, 1 female) served as untreated controls. Bodyweights of the animals ranged from 92.5 to 106.5 kg at the start of treatment and from 94.5 to 119 kg at slaughter. The 3 pigs on the 10 mg/kg treatment, plus 3 pigs (1 male, 2 female) from the 15 mg/kg treatment group, were killed at 12 hours after the last feeding, approximating "zero withdrawal". The remaining pigs on the 15 mg/kg treatment were killed in groups of 5 at 2 and 4 days after cessation of treatment with ractopamine. The results of residue analyses on the edible tissues from the animals in this study, based on a liquid chromatography method using electrochemical detection (limit of detection, 0.0015 mg/kg), are given in Table 4.

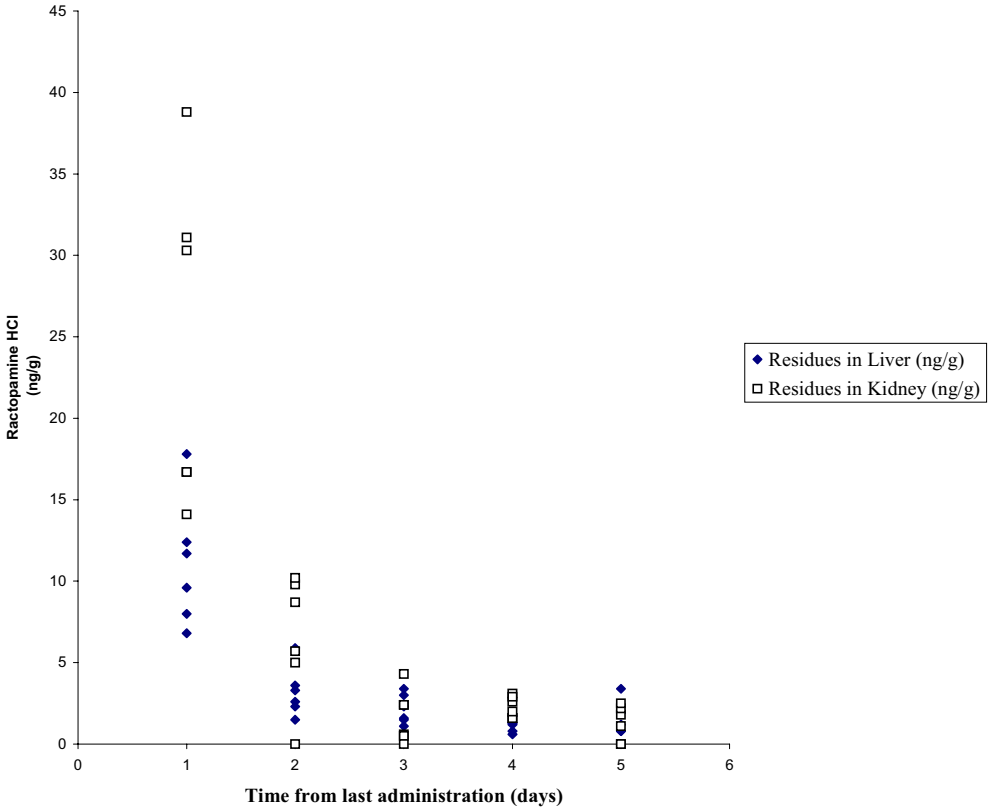
Table 4 Residues of ractopamine (parent compound) in tissues from pigs which received a ration containing either 10 mg/kg or 15 mg/kg of the drug in feed for 6 days.

Period following last treatment (days)	Treatment	n	Concentration of ractopamine (mg/kg)			
			liver	kidney	muscle	fat
0	control	1	0.0	0.0	0.0	0.0
0	10 mg/kg	3	0.012 ±0.005	0.020 ±0.008	0.003 ±0.001	0.0
0	15 mg/kg	3	0.026 ±0.008	0.045 ±0.011	0.005 ±0.001	0.001 ±0.000
2	15 mg/kg	5	0.005 ±0.002	0.006 ±0.003	---[a]	---[a]
4	15 mg/kg	5	0.001 ±0.000	0.002 ±0.001	---[a]	---[a]

[a] Not analyzed.

Another GLP study was conducted in which 30 pigs (15 male, 15 female; bodyweights 87-122 kg at slaughter) received a ration containing 20 mg/kg ractopamine hydrochloride for 9 days, after which groups of 6 pigs (3 male, 3 female) were killed at 1, 2, 3, 4 and 5 days after last ractopamine exposure (Turberg et al, 1991b). An additional 4 pigs (2 male, 2 female) received the ration without ractopamine hydrochloride and were used as controls. Livers and kidneys were collected at slaughter and analyzed for residues using liquid chromatography with electrochemical detection (limit of detection, 0.0005 mg/kg). No residues were detected in the control tissues. At 1 day after last administration, ractopamine residues were 0.011 ±0.004 mg/kg in livers and 0.025 ±0.010 mg/kg in kidneys, but had declined to 0.001 ±0.001 mg/kg in livers and 0.002 ±0.001 mg/kg in kidneys by day 5. Results of the analyses, in ng/g (1 ng/g = 0.001 mg/kg), are shown in Figure 5.

Figure 5 Residues in livers and kidneys of pigs fed a ration containing 20 mg/kg ractopamine hydrochloride for 9 days



A more recent GLP study was conducted in which 36 pigs (18 male, 18 female; 108-134 kg bw at slaughter) received a ration containing 20 mg/kg ractopamine hydrochloride for 10 days, after which groups of 6 pigs (3 male, 3 female) were killed at 12, 24, 36, 48, 60 and 72 hours after final ractopamine administration (Turberg et al, 1995). An additional 6 pigs (3 male, 3 female) received unmedicated ration and were a source of control tissue. Livers and kidneys were sampled at slaughter and analyzed for ractopamine using liquid chromatography with fluorescence detection. Results of the analyses, in ng/g (1 ng/g = 0.001 mg/kg) are shown in Figure 6.

Analysis of eyes from the control, 12 and 72-hour withdrawal animals demonstrated the presence of ractopamine residues (Martin, 2003). The mean concentrations were in the range of 200 ng/g for the retina + choroid + sclera and cornea + iris, 50 ng/g for the aqueous humor, and 10 ng/g for the lens. These were preliminary results only. A final audited report was not available at the time of this review.

An additional GLP depletion study was conducted in which 48 pigs (24 male, 24 female) were fed a ration containing 20 mg/kg ractopamine hydrochloride for 14 days, then slaughtered in groups of 8 (4 male, 4 female) at 12 hr and at 1, 2, 3, 4 and 5 days after last ractopamine administration (Donoho et al, 1991). Another 6 pigs (3 male, 3 female) received untreated feed and were a source of control tissues. At slaughter, samples of liver, kidney, muscle, fat and skin were collected from each animal and analyzed by liquid chromatography with electrochemical detection. The analytical results, given in Table 5, demonstrate the rapid depletion of residues in all the tissues, with highest residues found in kidney. Results of analysis of tissues of one animal from the 5-day group were eliminated as this pig became ill and lost weight during the course of the experiment.

Figure 6 Residues in livers and kidneys of pigs fed a ration containing 20 mg/kg ractopamine hydrochloride for 10 days

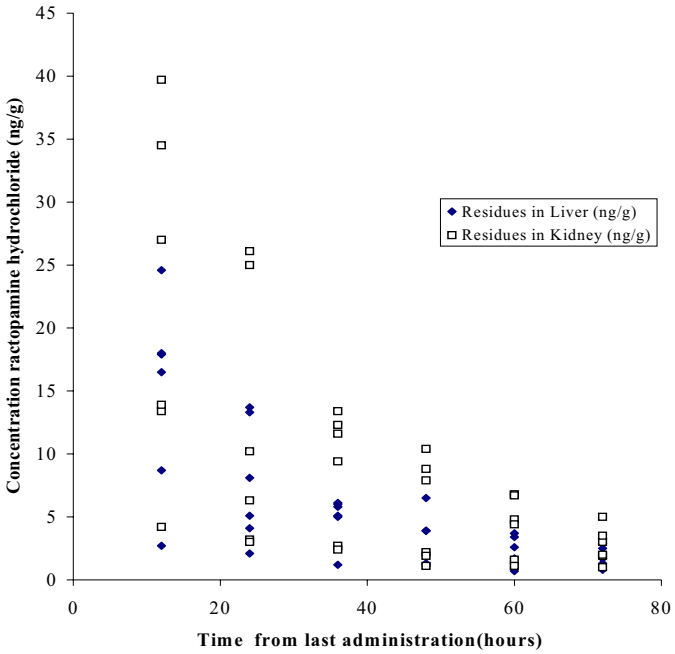


Table 5 Depletion of ractopamine residues in pigs fed a ration containing 20 mg/kg ractopamine hydrochloride for 14 days.

Time after last treatment (days)	n	Ractopamine residues in tissues (ng/g)				
		Liver	Kidney	Muscle	Fat	Skin
0	8	11.1 ±8.2	31.8 ±26.9	5.4 ±1.4 b	<2.0c	7.5 ±5.2
1	8	5.8 ±4.6	12.7 ±11.6	1.9 ±0.6	<1.0d	N.A. e
2	8	3.4 ±1.3	6.7 ±2.5	N.A.e	N.A. e	N.A. e
3	8	1.7 ±1.1	3.0 ±2.2	N.A. e	N.A. e	N.A. e
4	8	1.6 ±0.8f	2.2 ±1.5	N.A. e	N.A. e	N.A. e
5	7	<0.5a	<1.0 g	N.A. e	N.A. e	N.A. e

[a] No detectable residues (limit of detection 0.5 ng/g)
[b] Only tissues from animals with significant residues in liver and kidney were analyzed (Day 0, > 10 ng/g , n = 4; Day 1, > 5 ng/g, n = 4)
[c] Only two samples contained detectable residues (3.8 and 1.7 ng/g)
[d] Only two samples contained detectable residues (1.2 and 1.0 ng/g)
[e] Tissues not analyzed
[f] Two samples which contained no detectable residues were not included in the mean calculation
[g] Only two samples contained detectable residues (1.8 and 1.0 ng/g).

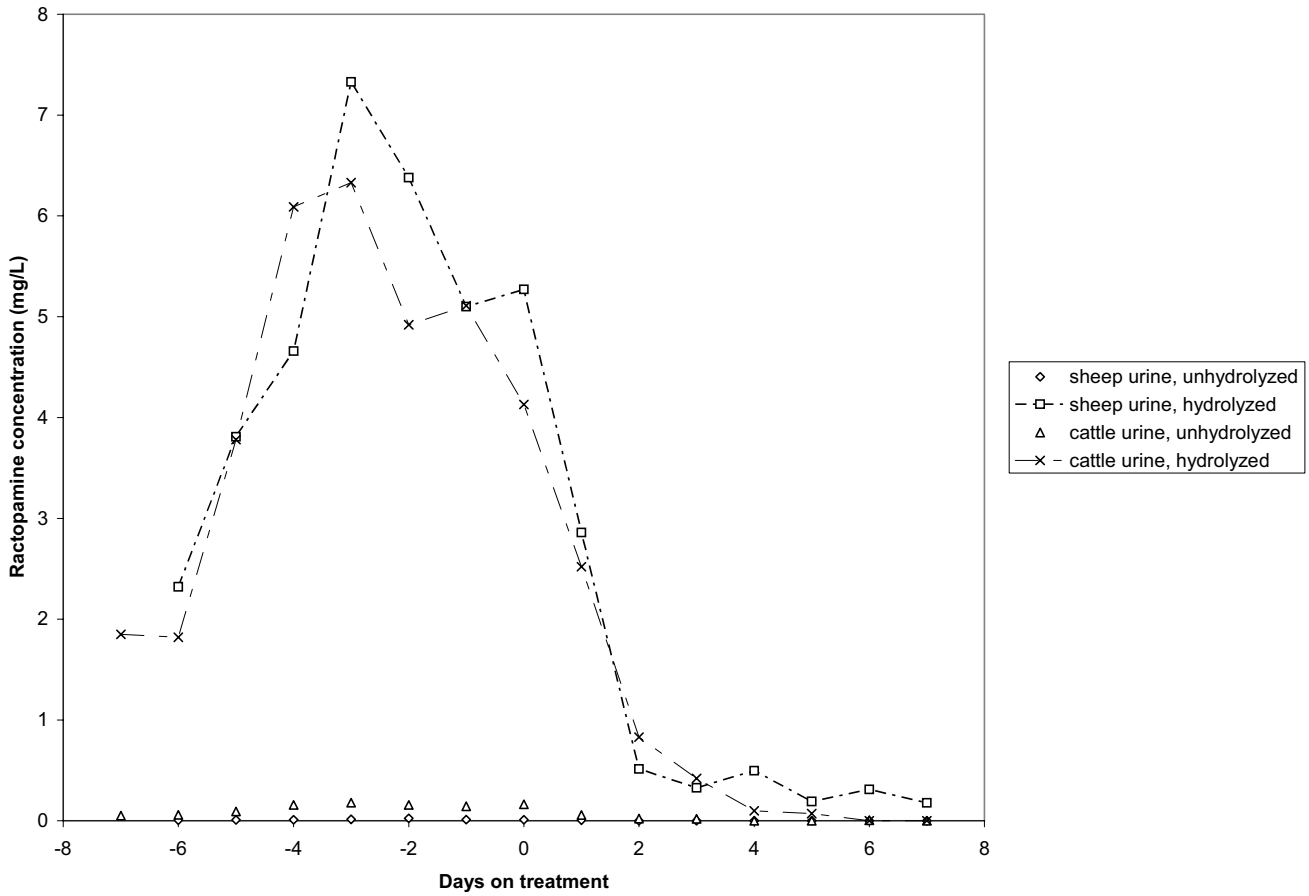
Cattle and other species

A GLP study was conducted in which 6 cattle (3 heifers, 3 steers) received feed containing 30 mg/kg ractopamine hydrochloride, 30 g/T monensin and 10 mg/T tylosin for 14.5 days (Moran & Buck, 1998). The animals were slaughtered 12 hr after the last administration and livers were analyzed for ractopamine by liquid chromatography. Mean concentrations expressed as ractopamine hydrochloride were 7.4 ± 3.1 ng/g. Treatment of a group of 6 heifers according to the same protocol, but with addition of melengesterol acetate to the feed at 0.5 mg/heifer/day, resulted in residues in liver of 4.1 ± 1.4 ng/g. The primary purpose of this study was to determine non-interference in the tissue residue depletion in cattle when ractopamine hydrochloride is used in combination with the other drugs. Animals used in this study were approximately 500 kg bw. The results were considered comparable with those obtained in previous trials where cattle received only radiolabeled ractopamine hydrochloride.

A non-GLP study was reported in which 6 heifers (315 ±21 kg) received a diet which included a concentrate containing 20 mg/kg ractopamine hydrochloride (equivalent to 0.43 mg/kg bodyweight per day) for 8 days, then were killed in pairs at 0, 3 and 7 days after cessation of treatment with ractopamine (Smith & Shelver, 2002). Urine was also collected from each animal before initial treatment and daily during the experiment. In the same study, 6 sheep (3 male, 3 female, bodyweight 75.7 ±8.4 kg) received a diet containing 20 mg/kg ractopamine hydrochloride (0.37 mg/kg bodyweight per day) for 7 days, then killed in pairs (1 male, 1 female) at 0, 3 and 7 days after cessation of ractopamine treatment. Urine was collected daily, beginning with the day prior to treatment. In addition, 9 ducklings (bodyweight 2.5 ±0.2 kg) received a diet containing 30 mg/kg for 7 days, after which the ducks were killed in groups of 3 at 0, 3 and 7 days after last treatment. Six ducks, which did not receive the treated feed, were used as a source of control tissue. The feeding periods used for all three species were to ensure a steady-state condition had been achieved. Residues in tissues were determined using a proposed regulatory method based on liquid chromatography with fluorescence detection (see Methods of Analysis for Residues in Tissues) which has a limit of quantification of 0.003 mg/kg estimated from the standard curve. Tissues extracts are cleaned up using an acidic alumina solid phase extraction procedure. For urine, the method was modified to use clean-up on a C-18 solid phase extraction cartridge. Conjugates were released from urine by hydrolysis with β-glucuronidase/aryl sulfatase from Patella vulgata after experiments using other enzymes demonstrated that this provided optimal results. Analysis of urine samples prior to and after hydrolysis demonstrated that the residues are predominantly as conjugates, as shown in Figure 7. These results were not corrected for recovery. Recoveries for both conjugated and unconjugated ratopamine residues averaged approximately 100% from sheep urine and 90% from cattle urine. Conjugated residues account for approximately 30 times the unconjugated residues in cattle urine and 400-600 times the unconjugated residues in sheep urine. The data suggest that treatment of cattle with ractopamine hydrochloride may be detectable in urine samples for up to 5 days after withdrawal of treatment, while treatment of sheep may be detected up to 7 days after last treatment, providing that samples are first hydrolyzed to release the conjugates.

In sheep, residues in liver and kidney were, respectively, 0.024 and 0.065 mg/kg on day 0, 0.003 mg/kg in the liver of one sheep at 3 days withdrawal and not detectable in the remaining liver and kidney samples. In cattle, residues in liver and kidney, respectively, were 0.009 and 0.098 mg/kg on day 0, 0.003 mg/kg in liver and kidney from one animal at day 3 and not detectable in the remaining tissue samples. No residues were detected in liver and kidney samples from the ducks. Subsequent analysis of the cattle and sheep livers using LC/MS/MS following enzymatic hydrolysis indicated that residues in the zero-withdrawal cattle and sheep livers were 0.028 and 0.064 mg/kg, respectively, suggesting that the parent ractopamine measured using the LC-fluorescence method represented 32% and 38%, respectively, of the total parent and metabolites present (Churchwell et al, 2002). The LC/MS/MS analysis also found ractopamine residues in retinal tissues of the cattle, ranging from 0.0005 to 0.0001 mg/kg, and from 0.0007 to 0.0031 mg/kg in retina from the sheep.

Figure 7 Excretion of ractopamine residues in urine of cattle and sheep during treatment and withdrawal



An evaluation of a number of commercial test kits designed for the detection of one or more beta-agonist compounds conducted in the mid-1990's demonstrated that none of the kits tested was suitable for the detection of ractopamine residues (Wicker et al, 1995). While these results do not necessarily apply to current versions of commercially available kits, they do demonstrate the need to carefully assess the performance of any kit, which may be considered for use in a regulatory program to ensure that it can detect residues of the target compounds at the required concentrations.

Subsequently, an ELISA procedure was reported for the detection of ractopamine residues in bovine urine (Elliot et al, 1998). Based on a polyclonal antibody, the test demonstrated little cross-reactivity to other β-agonists, including clenbuterol, salbutamol and isoxsuprine. Validation of the ELISA was conducted using 20 urine samples from calves not exposed to ractopamine as blank controls and also for experiments in which blanks were fortified at concentrations from 0.001 to 0.010 mg/L with ractopamine. Incurred samples from calves treated with ractopamine hydrochloride were also tested. It was also shown that enzyme treatment with ∃-glucuronidase derived from E. Coli and from H. pomatia gave similar results, approximately 2.5 times higher than from samples, which were not treated with enzyme to release the conjugates. The limit of detection, determined as three standard deviations from the mean response of blanks, was 0.002 mg/L, with intra-assay and inter-assay variability <13% at 0.002 – 0.010 mg/L. The method was used to detect ractopamine residues in animals which received a dose calculated as approximately 0.1 mg/kg bw per day over a period of 17 days, both during the dosing period and after withdrawal. The authors suggested that while monitoring of urine can be applied to detect ractopamine use for several weeks post-treatment, other matrices may be required to detect use after longer withdrawal times. Results were comparable to those obtained using an LC/MS/MS method, the details of which were reported in the same paper. A 5 mL test portion of urine was adjusted to pH 4.5 with 6M acetic acid, after the addition of deuterated clenbuterol and salbutamol as internal standard. Following the addition of 5 µL of β-glucuronidase (H. pomatia), test portions were incubated for 2 hr at 50 °C, then adjusted to pH 6.0 with 1 M sodium hydroxide solution and centrifuged at 1000 g for 10 min at 4 °C. The supernatants were then filtered (0.45 micron) and cleaned up with two types of solid phase extraction cartridges prior to LC/MS/MS analysis using atmospheric pressure chemical ionization (APCI). The ion fragments of ractopamine detected using selected reaction monitoring were the precursor ion (m/z 302) and product ions at m/z 164, 136, 121 and 107. Ion ratios used for confirmation were 107/164, 121/164 and 136/164 and were within 10% of those obtained from standards. The calibration curve covered the range 0.002 to 0.500 mg/L. Repeatability, determined at 0.003 and 0.006 mg/L, was within 10%.

An ELISA for ractopamine has also been reported which showed approximately 4% cross-reactivity with the phenylbutylamine glucuronides of the (RS, SR) diastereoisomers of ractopamine, little or no cross-reactivity with the (RR, SS) diastereoisomer glucuronides, other clenbuterol β-agonists such as clenbuterol and salbutamol, but cross-reactivity with dobutamine (Shelver & Smith, 2000). The authors reported that the method was suitable for detection of 0.001 mg/L ractopamine in urine.

Subsequently, an immunoaffinity column was reported for separation and clean-up of ractopamine residues from cattle urine and from bovine and sheep tissues (Shelver & Smith, 2002). The columns were tested on fortified samples of bovine urine, beef muscle, liver and kidney, and sheep muscle, liver and kidney. Recoveries were >80% from all tissues, with variability <10%, and extracts were suitable for LC analysis, with results comparable to those obtained using the proposed regulatory method with solid phase extraction cartridges. The columns demonstrated some stereospecificity, with potential for separating parent compound from metabolites.

Analytical methodology used in the initial residue depletion studies was based on liquid chromatography with electrochemical detection (Dalidowicz et al, 1986; Dalidowicz & Thomson, 1989). A 50 g test portion of ground or minced liver or kidney was blended with 75 mL of methanol for 1-2 min. and then transferred quantitatively to a 250 mL centrifuge bottle, using five washes of the blender jar (5 mL each). After centrifugation at 3500 rpm for 15 min., the supernatant was transferred into a 250 mL beaker and an additional 100 mL of methanol was added. The tissue pellet was re-suspended and the centrifugation was repeated. The combined supernatants were allowed to stand to settle precipitates, then a 4 mL aliquot was transferred to a test tube and the methanol was evaporated under a flow of nitrogen at 60 °C to a volume of about 0.5 mL. After addition of 5 mL water, the pH was adjusted to 10.5 ±0.5 by addition of 2M sodium carbonate, then 14 mL ethyl acetate was added. The mixture was shaken and, after phase separation, 10 mL of the ethyl acetate layer was transferred to a flask. The buffered sample was re-extracted with an additional 10 mL of ethyl acetate and a second 10 mL aliquot of the ethyl acetate layer was removed and combined with the initial extract. The combined extracts were evaporated to dryness, the residue was dissolved in 5 mL of acetonitrile/methanol (90:10) and loaded onto a silica solid phase extraction cartridge which had been pre-washed with 10 mL of acetonitrile/methanol (90:10). The flask was rinsed with two 3 mL portions of the acetonitrile/methanol load solution and added to the cartridge, which was then sequentially washed with 5 mL portions of load solution, methanol and dichloromethane. Ractopamine residues were eluted with 8 mL of dichloromethane/methanol/triethylamine (84:15:1), collected and evaporated to dryness. The residue was dissolved in 2 mL of mobile phase and a 25 :L aliquot was injected onto a C-18 column (4.6 mm x 25 cm, 5 micron particle) using a mobile phase of 0.05M ammonium phosphate buffer/acetonitrile (75:25) at a flow rate of 1 mL/min. An electrochemical detector was used to detect the residues of ractopamine by oxidation at a graphite electrode. A linear standard curve was obtained to cover the range 0.002 to 0.300 mg/L, with a limit of quantification estimated as 0.005 mg/kg. Analytical recoveries at 0.025 and 0.100 mg/kg ranged from 77-88% from fortified pig and cattle livers and kidneys, with precision <10%. No interferences or matrix effects were observed and sample extracts were stable for up to 6 days at 25 °C.

The proposed regulatory method provided by the sponsor for determinative analysis of residues in tissues is based on liquid chromatography with fluorescence detection (Moran & Turberg, 1998). The four stereoisomers co-elute as a single chromatographic peak and are expressed as ractopamine hydrochloride equivalents. In the initial method, developed for pig liver, kidney and muscle, a 10 gram test portion of tissue is homogenized methanol, then the mixture is centrifuged at 1500g for 10 min and the supernatant liquid is transferred to a flask. This step is repeated twice and the combined supernates are diluted to 60 mL with methanol. An 8 mL aliquot is reduced under nitrogen to < 0.5 mL. If concentrations >0.050 mg/kg are anticipated, the initial aliquot volume should be 2 mL. The residue is dissolved in borate buffer and ethyl acetate, centrifuged and the ethyl acetate layer is transferred into a small tube. This step is repeated. The two portions are added to an acidic alumina solid phase extraction cartridge which has been washed with 5 mL ethyl acetate. After a further wash with ethyl acetate, the ractopamine residues are eluted with methanol and dried. The residue is dissolved in 1 mL 2% acetic acid, filtered though a 0.45 micron syringe filter and a 100 :L aliquot is injected onto an LC column (25 cm x 4.6mm ID) packed with a 5 micron deactivated C-18 material. The mobile phase, prepared by mixing 320 mL acetonitrile, 680 mL water, 20 mL glacial acetic acid and 0.87 g 1-pentane sulfonic acid, is maintained at a flow rate of 1 mL/min, which elutes ractopamine in 4-8 minutes. Detection is by fluorescence, using an excitation wavelength of 226 nm and an emission wavelength of 305 nm. The concentration of ractopamine in the sample is calculated as ractopamine hydrochloride equivalents, with reference to a ractopamine hydrochloride standard curve, using the equation:

ng/g ractopamine hydrochloride = (A-B)/C x D x E/F

where

A = LC peak area of injected sample extract

B = intercept from the calibration curve

C = slope of the calibration curve (area/mL/ng)

D = purity of reference standard (g/g)

E = total volume (mL) = (initial volume/aliquot volume) x final volume

F = mass of tissue sample (g)

The above equation does not include a correction for recovery, as it is based on a calibration curve generated using standard solutions bracketing the appropriate range for the sample concentrations. It is recommended that a blank tissue, fortified at a concentration similar to that expected to be found in the samples, should be included in each analytical run, along with a tissue blank to provide an estimate of recovery.

Additional validation of the method for analysis of pig liver and kidney was conducted in the developer's laboratory, using a standard curve from 0.0025 to 0.100 mg/L (Turberg, 2001). A linear response was obtained over this range and this was not affected by the presence of matrix. Recoveries from tissues fortified at 0.020 to 0.200 mg/kg were 72-78% (relative standard deviation <11%) for muscle and 77-81% (relative standard deviation <10%) for liver. The limit of quantification was 0.002 (0.0018) mg/kg, determined as the lowest point on the calibration curve (0.0025 ng/g) times dilution/concentration factor (0.75). The method has also been validated for the analysis of residues in cattle tissues (Moran, 1998). Mean recoveries reported, based on 36 analyses per tissue type at concentrations from 0.002 to 0.020 mg/kg were 87% for muscle, 79% for fat, 75% for liver and 81% for kidney. Within day and between day variability (repeatability) is <15%, with a limit of quantification of 0.003 (0.0027) mg/kg.

An earlier version of the method using a curve from 0.0025 to 0.050 mg/L was validated in a multi-laboratory trial in which the developers and six additional laboratories tested the method on both fortified and incurred liver samples (Turberg et al, 1996). This study demonstrated successful transfer of the method to four of the six external participants, with between laboratory reproducibility <30%. One laboratory`s results, though in general agreement, were rejected due to deviation from the protocol, while another laboratory produced results approximately one-half the expected concentration on incurred samples which were attributed to a dilution error.

The confirmatory method proposed for regulatory use is based on LC/MS analysis of the extracts prepared for the determinative procedure (Kiehl, 1998). Following preparation of the initial extract, 4 replicate aliquots are processed through to elution of ractopamine residues from the solid phase extraction cartridge with methanol, instead of the single 8 mL aliquot required in the determinative method. After elution from the SPE cartridge, the eluates from the four replicates are each reduced to approximately 1 mL in volume and combined, then evaporated to dryness. The residue is dissolved in 0.200 mL 0.01M ammonium acetate (pH 4.5) and a 15 µL aliquot in injected into the LC/MS system. The analytical column (30 cm x 1 mm I.D.) is packed with a deactivated C-18, 5 micron, material. A flow rate of 0.10 mL/min of mobile phase (0.01M ammonium acetate, pH 4.5/acetonitrile, 82:18) is used, with a pneumatically assisted electrospray direct interface (no flow splitting). Ractopamine is detected in the positive ion mode, using selected ion monitoring for the ions with mass/charge (m/z) ratios 302, 284 and 164. The ions monitored are the protonated parent and two fragments. The fragment with m/z 164 has been attributed to cleavage at the amino group in the chain joining the aromatic rings in the parent structure. Fragment 284 corresponds to a loss of water. The method was successfully tested on both fortified and incurred liver and muscle samples, using a requirement that ion ratios 284/302, 164/302 and 164/284 show agreement between samples and standards within 10%. An earlier version of the method, in which the final extract for LC/MS analysis was taken up in 0.200 mL methanol/water

(50:50) was subjected to a multi-laboratory trial, the complete results of which were not available for review by the Committee (Turberg, Buck, Geroulis & Kiehl, 1996).

Use of LC/MS/MS methodology has also been reported for the detection of ractopamine residues in pork liver, kidney and muscle, plus lung and retinal tissues, as well as bovine urine (Antignac et al., 2002). Samples were freeze-dried, ground and, after an initial extraction with methanol and acetate buffer, the methanol was removed by evaporation and the buffered extract was incubated with β-glucuronidase (H. pomatia) for 15 hr at 60 °C. Extracts were then cleaned up using two solid phase extraction cartridges, the eluate was evaporated to dryness and taken up in 50 µL of 0.5% acetic acid in water/methanol (97:3). Ractopamine was monitored using the precursor ion m/z 302, plus the fragment ions with m/z 284, 164, 136, 121, 107 and 91. Using isoxsuprine as an internal standard, the method was validated to meet current EU performance criteria for confirmatory methods, with a decision limit of 10 ng/kg and a detection capability of 30 ng/kg.

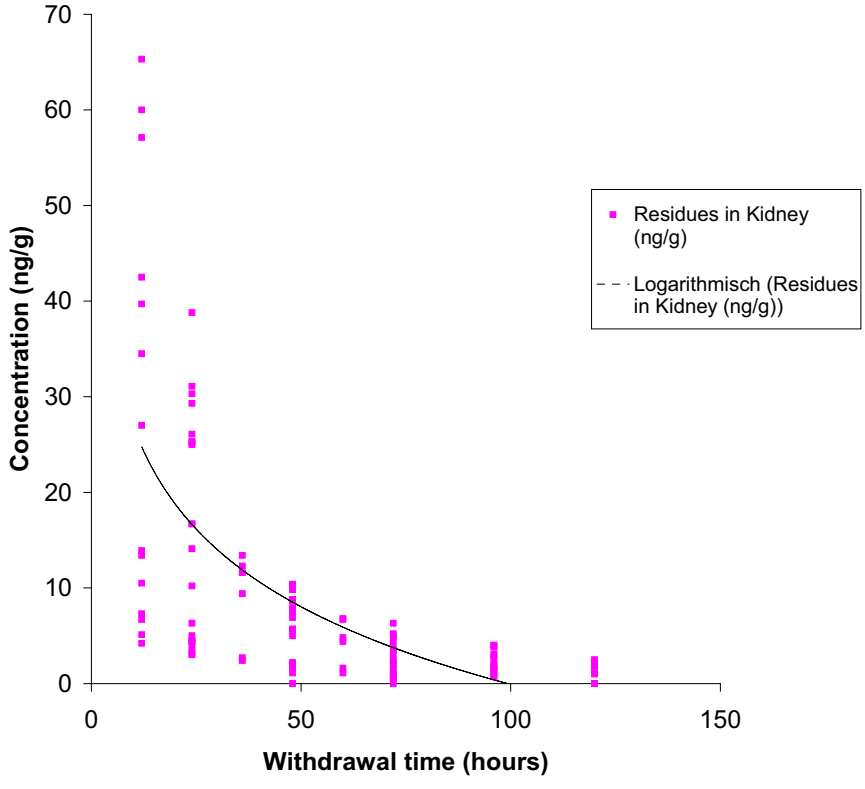
More recently, a method has been published for the detection of ractopamine residues in pork and beef muscle, using LC-fluorescence and LC/MS/MS (Shishani et al, 2003). This method includes an initial extraction with methanol, incubation with β-glucuronidase (*H. pomatia*) at 65oC for 2 hr, then extraction into ethyl acetate after addition of borate buffer. The ethyl acetate extract is cleaned up on an alumina solid phase extraction cartridge and ractopamine is eluted with methanol, evaporated to dryness and the residue is dissolved in 1M acetic acid. This solution is then further cleaned up using an ion exchange cartridge (Oasis SPE MCX, 6mL, 500 mg) and ractopamine is eluted with 2% ammonia in methanol. The eluate is taken to dryness, dissolved in 0.5 mL 2% acetic acid and a 0.100 mL aliquot is injected into the LC. The column and mobile phase are as described in the proposed regulatory method, described above. Quantitative determination is by fluorescence detection (excitation, 226 nm; emission, 306 nm) using ritodrine as an internal standard. For confirmatory analyses, the dried extract from the ion exchange SPE cartridge is taken up in methanol and analyzed by LC/MS/MS using a reversed phase C-16 amide column packing. Ions monitored for ractopamine were m/z 302, 164, 121 and 107. Recoveries of 80-117% and 85-114% were reported for pork and beef muscle, respectively, at concentrations from 0.001 to 0.004 mg/kg.

APPRAISAL

All the laboratory and food animal species studied metabolized ractopamine through glucuronidation. Three chromato-graphically distinct monoglucuronides, designated as metabolites A, B and C, were present in liver and kidney tissues and urine of all species studied and were identified by means of fast atom bombardment (FAB) mass spectrometry and nuclear magnetic resonance (NMR). The metabolite A consists of isomers RS and SR and the metabolite B of isomers RR and SS. The Metabolite C is a mixture. Metabolite D, a major metabolite in cattle, also found in other species studied was characterized as a diglucuronide.

The metabolism and pharmaco-kinetics studies were all performed using ^{14}C-ractopamine and most studies were in compliance with the US FDA and OECD Good Laboratory Practice (GLP) standards. The analytical procedures used were largely identical in all studies. The initial extraction from tissues was performed using NH_4HCO_3 at pH 10. Following extractions with organic solvents and Amberlite treatment the solution was further extracted with diethyl ether. The aqueous and organic phases were then subjected to reversed and normal phase liquid chromatography. Fraction of the column effluent was collected and subjected to determination of radioactivity of each appropriate fraction. Structural information was also obtained from these fractions.

Figure 8. Residues of ractopamine in swine kidney (ng/g) - Pooled data: studies using ration containing 20 mg/kg ractopamine hydrochloride



Residue studies were provided using both labeled and unlabeled ractopamine hydrochloride for both swine and cattle and most studies were in compliance with contemporary GLP standards. As in the pharmacokinetic and metabolism studies, the analytical methodology used was similar in most studies, although some studies used liquid

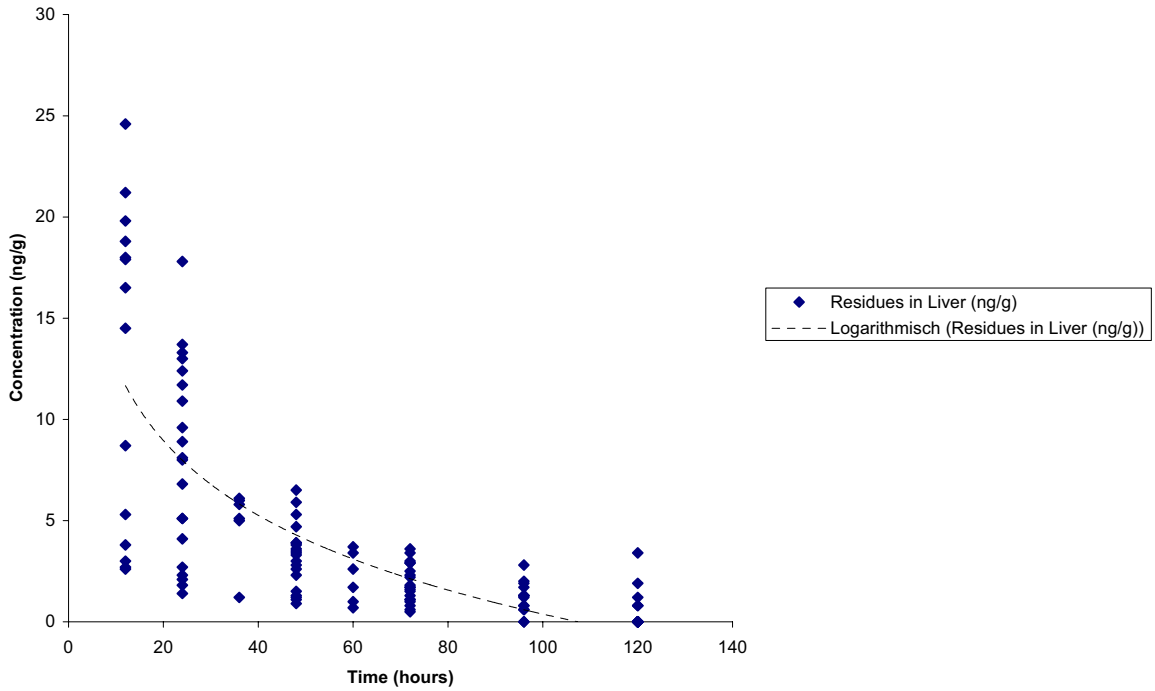
chromatography with electrochemical detection and later studies used fluorescence detection, which has been proposed as the regulatory method.

Absorbance and excretion of ractopamine is rapid, with concentrations in both swine and cattle reaching a steady state within 4 days of the start of treatment with medicated feed. Residues are detectable in urine using contemporary analytical methods for a week or longer following withdrawal of the drug, but are primarily present as glucuronides. Residues are found primarily in liver and kidney tissues and are near or below detection limits in muscle and fat at the start of withdrawal. Highest residues are found in the kidney in pigs and in cattle, but are at or below the 1 ng/g (1 µg/kg or 0.001 mg/kg) range within 3-7 days of withdrawal. Residues are more persistent in retinal tissue. Based on the information provided, the recommended target tissue for residue monitoring where use of ractopamine has been approved is kidney. The depletion of ractopamine residues in swine kidney (Figure 8) and liver (Figure 9), based on pooling of data from the studies conducted using the maximum recommended concentration of ractopamine hydrochloride in feed (20 mg/kg feed), demonstrates the rapid elimination of the residues.

The mean residue of ractopamine hydrochloride in swine kidney at slaughter 12 hr post-administration from the pooled data in Figure 6 was 27.6 ±22.1 ng/g (µg/kg), while in liver the mean concentration at this time was 12.7 ±7.9 ng/g (µg/kg). Correcting from ractopamine hydrochloride to ractopamine free base using a factor of 0.89 and adding three standard deviations provides estimated maximum concentrations for MRLs of 90 µg/kg for kidney and 40 µg/kg for liver.

Residues found in muscle and fat tissue were much lower than those reported in kidney and liver, ranging from non-detectable to maximum mean free ractopamine concentrations (as hydrochloride equivalents) of 5.4 ±1.4 µg/kg in muscle and <2.0 µg/kg in fat at 12 hours post-administration. These residues were similar to the highest total residues reported at 12 hr post-administration in studies with radiolabeled ractopamine hydrochloride. Using the highest reported residues (5.4 ±1.4 µg/kg) of

Figure 9. Residues of ractopamine in swine liver (ng/g) - Pooled data: studies using ration containing 20 mg/kg ractopamine hydrochloride



ractopamine hydrochloride equivalents, converting to ractopamine base and adding three standard deviations results in a maximum estimate for MRLs for muscle of 8.5 µg/kg. However, it was also noted that some residue studies were conducted using a liquid chromatographic assay with a limit of quantification of 5 µg/kg for analysis of muscle and fat. While subsequent work demonstrated limits of quantification in the range of 2 µg/kg for analysis of muscle, the higher limit of quantification provides a more conservative estimate of detection capability, given the differences in performance of chromatographic systems and, in particular, chromatographic detectors. Therefore, using twice an LOQ of 5 µg/kg for the analysis of muscle and fat encompasses the upper estimate derived from the maximum residue concentrations reported in muscle samples at 12 hr post-administration. It also provides for the possibility that some detectable residues might have been reported in some fat samples had a method with an LOQ of 2 µg/kg been used in the residue studies conducted at an LOQ of 5 µg/kg. Finally, achievement of an LOQ of 5 µg/kg for muscle and fat should be within the capabilities of residue control laboratories equipped with a liquid chromatograph using either electrochemical or fluorescence detection, as reported in the residue depletion and method validation studies considered by the Committee. However, since detectable residues of ractopamine were reported in some muscle and fat samples at 12 hr post-administration, it is not appropriate to treat the MRLs based on twice the LOQ as merely advisory in this situation. Instead, the MRLs for muscle and fat should be used in estimating a theoretical maximum daily intake. This is not the same as the situation for some other substances which have been reviewed by the Committee,

where no detectable residues were reported in any depletion studies in certain tissues. In the case of ractopamine, residues have been reported, but usually at or below the limit of quantification in muscle and below the limit of quantification in fat.

Suitably validated methods have been provided for the determination and confirmation of ractopamine residues in edible tissues of swine and cattle. The methods include liquid chromatography with fluorescence detection for detection and determination and liquid chromatography with mass spectrometry detection for confirmation. The method requirements are within the capabilities of most well-equipped residue control laboratories.

MAXIMUM RESIDUE LIMITS

In recommending MRL's, the Committee took into account the following factors:

- An ADI of 0-1 μg per kg of body weight was established by the Committee, equivalent to 0-60 μg for a 60 kg person. The parent compound, ractopamine, is the appropriate marker residue.
- The appropriate target tissue for a routine monitoring program is kidney.
- Suitable analytical methods are available for analysis of ractopamine residues in edible tissues of pigs and cattle.
- Animals which have been treated with ractopamine will usually be slaughtered within 12 to 24 hr of consumption of feed containing ractopamine hydrochoride, so Maximum Residue Limit calculations are based on tissue residues at 12 hr post-administration.
- Maximum residue limits for liver and kidney of pigs and cattle were based on the mean residue concentrations of free ractopamine plus 3 standard deviations. The mean was calculated from the pooled data for pigs in all studies at 12 hr following the last feeding at the maximum recommended dose, 20 mg/kg. These were higher than the free ractopamine residues observed in cattle liver and kidney at 12 hr post-administration. Factors to convert free ractopamine to total residues are 5 for liver and 6 for kidney of pigs and cattle. The factors derived at 12 hr following the last feeding are based on the results obtained in cattle, which provides a more conservative estimate of exposure.
- The Maximum Residue Limits for muscle and fat were based on twice the LOQ of 0.005 μg/kg. A correction factor to convert marker to total residues was not required.

On the basis of the above considerations, the Committee recommended the following MRL's for edible tissues of pigs and cattle, expressed as ractopamine base: for muscle 10 μg/kg, for liver 40 μg/kg, for kidney 90 μg/kg, and for fat 10 μg/kg.

The MRL's recommended above would result in a theoretical daily maximum intake of 50.5 μg, based on a daily food intake of 300 g of muscle, 100 g of liver, 50 g each of kidney and fat, or 84% of the upper bound of the acceptable daily intake.

Estimates of residue intake are tabulated as follows:

Table 6 Theoretical Maximum Daily Intake (TMDI) of Ractopamine Residues

Food Item	MRL (μg/kg)	Food Basket (kg)	MR/TR[1]	TMDI (μg)
Muscle	10	0.300	1	3.0
Liver	40	0.100	5	20.0
Kidney	90	0.050	6	27.0
Fat	10	0.050	1	0.5
Total:				50.5

[1] MR = marker residue (parent drug); TR = total residues

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SUMMARY OF JECFA EVALUATIONS OF VETERINARY DRUG RESIDUES FROM THE 32ND MEETING TO THE PRESENT

This following table summarises the veterinary drug evaluations conducted by JECFA at the 32nd (1987), 34th (1989), 36th (1990), 38th (1991), 40th (1992), 42nd (1994), 43rd (1994), 45th (1995), 47th (1996), 48th (1997), 50th (1998), 52nd (1999), 54th (2000), 58th (2002), 60th (2003) and 62nd (2004) meetings. These meetings were devoted exclusively to the evaluation of veterinary drug residues in food. **This table must be considered in context with the full reports of these meetings, which are published as WHO Technical Report Series.**

Some notes regarding the Table:

- The "ADI Status" column refers to the ADI and indicates whether an ADI was established, if a full ADI was given, or if the ADI is temporary (T).
- Where an MRL is temporary, it is so indicated by "T".
- Several compounds have been evaluated more than once. The data given are for the most recent evaluation, including the 60th meeting of the Committee.

A comprehensive listing of references to all JECFA evaluations and publications is available from the on-line edition of the *Summary of Evaluations Performed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA 1956-2001)* which can be accessed from FAO and WHO websites for JECFA (www.fao.org/es/esn/jecfa/index_en.stm and www.who.int/ipcs/food/jecfa/en/).

Substance	ADI (µg/kg bw)	ADI Status	JECFA	MRL (µg/kg)	Tissue	Species	Marker residue and other remarks
Abamectin	0-1 (1995 JMPR)	Full	47 (1996)	100 50	Liver, fat Kidney	Cattle	Avermectin B_{1a}
Albendazole	0-50	Full	34 (1989)	100 5000	Muscle, fat, milk Liver, kidney	Cattle, sheep	MRLs analysed as 2-amino-benzimidazole and expressed as parent drug equivalents, see WHO TRS 788
Azaperone	0-6	Full	50 (1998)	60 100	Muscle, fat Liver, kidney	Pigs	Sum of azaperone and azaperol
Benzylpenicillin	30 µg/person/day	Full	36 (1990)	50 4	Muscle, liver, kidney Milk	All species	Parent drug
Bovine Somatotropins	Not specified	Full	50 (1998)	Not specified	Muscle, liver, kidney, fat, milk	Cattle	
Carazolol	0-0.1	Full	43 (1994)	5 25	Muscle, fat/skin Liver, kidney	Pigs	Parent drug. The Committee noted that the concentration of carazolol at the injection site may exceed the ADI which is based on the acute pharmacological effect of carazolol
Carbadox	No ADI		60 (2003)	No MRL			Quinoxaline-2-carboxylic acid
Ceftiofur	0-50	Full	45 (1995) 48 (1997)	 1000 2000 6000 2000 100 µg/l	 Muscle Liver Kidney Fat Milk	 Cattle, pigs Cattle	 Desfuroylceftiofur
Cefuroxime	No ADI		62 (2004)	No MRL			
Chloramphenicol	No ADI		62 (2004)	No MRL			
Chlorpromazine	No ADI		38 (1991)	No MRL			
Chlortetracycline, oxytetracycline, tetracycline	0-30 (Group ADI)	Full	58 (2002)	200 600 1200 400 100 µg/l 100 200	Muscle Liver Kidney Eggs Milk Muscle Muscle	Cattle, pigs, sheep, poultry Poultry Cattle, sheep Giant prawn Fish	Parent drugs, singly or in combination Oxytetracycline only

Substance	ADI (µg/kg bw)	ADI Status	JECFA	MRL (µg/kg)	Tissue	Species	Marker residue and other remarks
Clenbuterol	0-0.004	Full	47 (1996)	0.2 0.6 0.05 µg/l	Muscle, fat Liver, kidney Milk	Cattle, horses Cattle	Parent drug
Closantel	0-30	Full	36 (1990) 40 (1992)	 1000 3000 1500 5000 2000	 Muscle, liver Kidney, fat Muscle, liver Kidney Fat	 Cattle Sheep	Parent drug
Cyfluthrin	0-20	Full	48 (1997)	20 200 40 µg/l	Muscle, liver, kidney Fat Milk	Cattle	Parent drug
Cyhalothrin	0-2	T	62 (2004)	50 1000 100	Muscle, liver, kidney Fat Milk	Cattle, pig, sheep Cattle, sheep	Parent drug
Cypermethrin	0-20	Full	62 (2004)	50 1000 100	Muscle, liver, kidney Fat Milk	Cattle, sheep Cattle, sheep	Total of cypermethrin residues (resulting from the use of cypermethrin or alpha-cypermethrin as veterinary drugs)
α-Cypermethrin	0-20	Full	62 (2004)	50 1000 100	Muscle, liver, kidney Fat Milk	Cattle, sheep Cattle, sheep	Total of cypermethrin residues (resulting from the use of cypermethrin or alpha-cypermethrin as veterinary drugs)
Danofloxacin	0-20	Full	48 (1997)	200 400 100 100 50 200 100	Muscle Liver, kidney Fat Muscle Liver Kidney Fat	Cattle, chickens Pigs	Parent drug For chickens fat/skin in normal proportion
Deltamethrin	0- 10 (JMPR 1982)	Full	60 (2003)	30 50 500 30 30	Muscle Liver, kidney Fat Milk Egg	Cattle, sheep, chicken, salmon Cattle, sheep, chicken Cattle, sheep, chicken Cattle Chicken	Parent drug

Substance	ADI (µg/kg bw)	ADI Status	JECFA	MRL (µg/kg)	Tissue	Species	Marker residue and other remarks
Dexamethasone	0-0.015	Full	50 (1998)	No MRL			Temporary MRLs were not extended Regulatory method not available
Diclazuril	0-30	Full	50 (1998)	500 3000 2000 1000	Muscle Liver Kidney Fat	Sheep, rabbits, poultry	Parent drug Poultry skin/fat
Dicyclanil	0-7	Full	60 (2003)	150 125 200	Muscle Liver, kidney Fat	Sheep	Parent drug
Dihydrostrepto-mycin, streptomycin	0-50 (Group ADI)	Full	58 (2002)	600 1000 200	Muscle, liver, fat Kidney Milk	Cattle, pigs, sheep, chickens Cattle, sheep	Sum of dihydrostreptomycin and strepto-mycin
Demetridazole	No ADI		34 (1989)	No MRL			
Diminazene	0-100	Full	42 (1994)	500 12000 6000 150 µg/l	Muscle Liver Kidney Milk	Cattle	Parent drug
Doramectin	0-0.5	Full	62 (2004)	10 5 100 30 150 15	Muscle Muscle Liver Kidney Fat Milk	Cattle Pigs Cattle, Pigs Cattle	Parent drug
Enrofloxacin	0-2	Full	48 (1997)	No MRL			
Eprinomectin	0-10	Full	50 (1998)	100 2000 300 250 20 µg./l	Muscle Liver Kidney Fat Milk	Cattle	Eprinomectin B_{1a}
Estradiol-17β	0-0.05	Full	52 (1999)	Not specified	Muscle, liver, kidney, fat	Cattle	
Febantel, fenbendazole, oxfendazole	0-7 (Group ADI)	Full	50 (1998)	100 500 100 µg/L	Muscle, kidney, fat Liver Milk	Cattle, sheep, pigs, horses, goats Cattle, sheep	Sum of fenbendazole, oxfendazole, and oxfendazole sulfone, expressed as ox-fendazole sulfone equivalents

Substance	ADI (µg/kg bw)	ADI Status	JECFA	MRL (µg/kg)	Tissue	Species	Marker residue and other remarks
Fenbendazole (see febantel)							
Fluazuron	0-40	Full	48 (1997)	200 500 7000	Muscle Liver, kidney Fat	Cattle	Parent drug
Flubendazole	0-12	Full	40 (1992)	10 200 500 400	Muscle, liver Muscle Liver Eggs	Pigs Poultry	Parent drug
Flumequine	0-30	Full	62 (2004)	500 1000 500 3000 500 500 T	Muscle Fat Liver Kidney Muscle Muscle	Cattle, sheep pigs, chicken Trout Black Tiger shrimp (*P.monodon*)	Parent drug
Furazolidone	No ADI		40 (1992)	No MRL			
Gentamicin	0-20	Full	50 (1998)	100T 2000 5000 200 µg/l	Muscle, fat Liver Kidney Milk	Cattle, pigs Cattle	Parent drug
Imidocarb	0-10	Full	60 (2003)	300 1500 2000 50	Muscle Liver Kidney Fat, milk	Cattle	Parent drug
Ipronidazole	No ADI		34 (1989)	No MRL			
Isometamidium	0-100	Full	40 (1992)	100 500 1000	Muscle, fat, milk Liver Kidney	Cattle	Parent drug
Ivermectin	0-1	Full	58(2002)	100 40 15 20 10	Liver Fat Liver Fat Milk	Cattle Pigs, sheep Cattle	Ivermectin B_{1a}

Substance	ADI (µg/kg bw)	ADI Status	JECFA	MRL (µg/kg)	Tissue	Species	Marker residue and other remarks
Levamisole	0-6	Full	42 (1994)	10 100	Muscle. Kidney, fat Liver	Cattle, sheep, pigs, poultry	Parent drug
Lincomycin	0-30	Full	58 (2002)	200 500 1500 500 100 150	Muscle Liver Kidney " Fat Milk	Chickens, pigs " Pigs Chicken Chickens, pigs Cattle	Parent drug A separate MRL of 300 µg/kg for skin with adhering fat in pigs was recommended in order to reflect the high concentrations found in the skin of pigs. For consistency, an MRL of 300 µg/kg for skin with adhering fat in chickens was also recommended.
Melengestrol acetate	0-0.03	Full	62 (2004)	5 8	Liver Fat	Cattle	Parent drug
Metronidazole	No ADI		34 (1989)	No MRL			
Moxidectin	0-2	Full	50 (1998)	100 50 500 50 20 20 100 50 500	Liver Kidney Fat Muscle Muscle Muscle Liver Kidney Fat	Cattle, sheep Sheep Cattle Deer	Parent drug. The Committee noted the very high concentration and great variation in the level of residues at the injection site in cattle over a 49-day period after dosing.
Neomycin	0-60	Full	60 (2003)	500 500 10000 500 1500	Muscle, fat Liver Kidney Eggs Milk	Cattle, chicken, duck, goat, pig, sheep, turkey Cattle, chicken, duck, goat, pig, sheep, turkey Cattle, chicken, duck, goat, pig, sheep, turkey Chicken Cattle	Parent drug
Nicarbazin	0-400	Full	50 (1998)	200	Muscle, liver, kidney, fat/skin	Chicken (broilers)	

Substance	ADI (µg/kg bw)	ADI Status	JECFA	MRL (µg/kg)	Tissue	Species	Marker residue and other remarks
Nitrofurazone	No ADI		40 (1992)	No MRL			
Olaquindox	Limited acceptance	T	42 (1994)	No MRL (see remarks)	Muscle	Pigs	MQCA[1]. The Committee recommended no MRLs but noted that 4 µg/kg of MQCA (T) is consistent with Good Veterinary Practice
Oxfendazole (see febantel)							
Oxolinic acid	No ADI		43 (1994)	No MRL			
Oxytetracycline (see chlortetracycline)							
Permethrin	No ADI		54 (2000)	No MRL			
Phoxim	0-4	Full	62 (2004)	50 400	Muscle, liver, kidney Fat	Goats, pigs, sheep	Parent drug
Pirlimycin	0-8	Full	62 (2004)	100 1000 400	Muscle, fat, milk Liver Kidney	Cattle	Parent drug
Porcine somatotropins	Not specified		52 (1999)	Not specified	Muscle, liver, kidney, fat	Pigs	
Procaine benzylpenicillin	Less than 30 µg of penicillin per person per day	Full	50 (1998)	50 4 µg/kg	Muscle, liver, kidney Milk	Cattle, pigs, chickens Cattle	Benzylpenicillin
Progesterone	0-30	Full	52 (1999)	Not specified	Muscle, liver, kidney, fat	Cattle	
Propionyl-promazine	No ADI		38 (1991)	No MRL			
Ractopamine	0-1	Full	62 (2004)	10 40 90	Muscle, fat Liver Kidney	Cattle, pigs	Parent drug
Ronidazole	No ADI		42 (1994)	No MRL			
Sarafloxacin	0-0.3	Full	50 (1998)	10 80 20	Muscle Liver, kidney Fat	Chicken, turkey Chicken, turkey Chicken, turkey	Parent drug

[1] MQCA: 3-methylquinoxaline-2-carboxylic acid

Substance	ADI (µg/kg bw)	ADI Status	JECFA	MRL (µg/kg)	Tissue	Species	Marker residue and other remarks
Spectinomycin	0-40	Full	50 (1998)	500 2000 5000 2000 200 µg/kg	Muscle Liver, fat Kidney Eggs Milk	Cattle, pig, sheep, chicken Chicken Cattle	Parent drug
Spiramycin	0-50	Full	48 (1997)	200 600 300 800 300 200 µg/kg	Muscle Liver Kidney Kidney Fat Milk	Cattle, chicken, pig Cattle, chicken, pig Cattle, pig Chicken Cattle, chicken, pig Cattle	For cattle and chickens MRLs are expressed as the sum of spiramycin and neospiramycin For pigs MRLs expressed as spiramycin equivalents (antimicrobially active residues)
Streptomycin (see dihydrostrepto-mycin)							
Sulfadimidine	0-50	Full	42 (1994)	100 25 µg/kg	Muscle, liver, kidney, fat Milk	Cattle, sheep, pig, poultry Cattle	Parent drug
Sulphthiazole	No ADI		34 (1989)	No MRL			
Testosterone	0-2	Full	52 (1952)	Not specified	Muscle, liver, kidney, fat	Cattle	
Tetracycline (see Chlortetracycline)							
Thiamphenicol	0-5	Full	58 (2002)	No MRl			
Tiabendazole	0-100	Full	58 (2002)	100 100 µg/kg	Muscle, liver, kidney, fat Milk	Cattle, pig, goat, sheep Cattle, goat	Sum of thiabendazole and 5-hydroxythiabendazole
Tilmicosin	0-40	Full	47 (1996)	100 1000 1500 300 1000 50 µg/kg T	Muscle, fat Liver Liver Kidney Kidney Milk	Cattle, pig, sheep Cattle, sheep Pig Cattle, sheep Pig‘ Sheep	Parent drug
Trenbolone acetate	0-0.02	Full	34 (1989)	2 10	Muscle Liver	Cattle	β-Trenbolone for muscle α-trenbolone for liver

Substance	ADI (µg/kg bw)	ADI Status	JECFA	MRL (µg/kg)	Tissue	Species	Marker residue and other remarks
Trichlorfon (Metrifonate)	0-2	Full	60 (2003)	50 µg/kg 50	Milk Muscle, liver, kidney, fat	Cattle Cattle	Parent drug Guidance MRLs (No residues detected in depletion studies. No residues should be present in tissues when used with good veterinary practice. Limit of quantification used as guideline MRL)
Triclabendazole	0-3	Full	40 (1992)	200 300 100 100	Muscle Liver, kidney Fat Muscle, liver kidney, fat	Cattle Sheep	5-Chloro-6-(2',3'-dichlorophenoxy)-benzimidazole-2-one
Tylosin	No ADI		38 (1991)	No MRL			
Xylazine	No ADI		47 (1996)	No MRL			
Zeranol	0-0.5	Full	32 (1987)	2 10	Muscle Liver	Cattle	Parent drug

ANNEX 2

Summary of Recommendations from the 62nd JECFA on Compounds on the Agenda and Further Information Required

Antimicrobial agents

Cefuroxime

Acceptable daily intake: The temporary ADI established at the fifty-eighth meeting of the Committee (WHO TRS 911, 2002) was withdrawn.

Residues: The temporary MRL for cattle milk was withdrawn.

Chloramphenicol

Acceptable daily intake: The Committee concluded that it is not appropriate to establish an ADI for chloramphenicol.

Residues: The Committee concluded that

There was no evidence supporting the hypothesis that chloramphenicol is synthesized naturally in detectable amounts in soil. Although this possibility is highly unlikely, data generated with modern analytical methods would be required to confirm this;

There was evidence that low concentrations of chloramphenicol found in food monitoring programs in the year 2002 could not originate from residues of chloramphenicol persisting in the environment after historical veterinary uses of the drug in food producing animals. However, due to the high variability of the half life of chloramphenicol under different environmental conditions, such a mechanism might occasionally cause low level contamination in food;

Valid analytical methods are available to monitor low levels of chloramphenicol in foods. However confirmatory methods require sophisticated and expensive equipment.

Flumequine

Acceptable daily intake: The Committee re-established an ADI of 0–30 µg/kg bw.

Residue definition: Flumequine

Recommended maximum residue limits (MRLs)

Species	Fat (µg/kg)	Kidney (µg/kg)	Liver (µg/kg)	Muscle (µg/kg)
Cattle	1000	3000	500	500
Black tiger shrimp (*P. monodon*)	-	-	-	500[a]
Chicken	1000	3000	500	500
Pigs	1000	3000	500	500
Sheep	1000	3000	500	500
Trout	-	-	-	500[b]

[a] The MRL is temporary; the following information is requested by 2006: (1) A detailed description of a regulatory method, including its performance characteristics and validation data; (2) Information on the approved dose for treatment of black tiger shrimp and the results of residue studies conducted at the recommended dose.

[b] Muscle including normal proportions of skin.

Lincomycin

Acceptable daily intake: 0-30 μg/kg bw (established at the fifty-fourth meeting of the Committee (WHO TRS 900, 2001)

Residues: The MRLs that were recommended by the fifty-fourth (WHO TRS 900, 2001) and fifty eighth (WHO TRS 911, 2002) meeting of the Committee were not reconsidered and maintained. MRL for cattle tissues were considered but not recommended by the sixty-second meeting.

Pirlimycin

Acceptable daily intake: The Committee established an ADI of 0–8 μg/kg bw

Residue definition: Pirlimycin

Recommended maximum residue limits (MRLs)[a]

Species	Fat (μg/kg)	Kidney (μg/kg)	Liver (μg/kg)	Milk (μg/kg)	Muscle (μg/kg)
Cattle	100	400	1000	100	100

[a] For the Maximum Residue Limits for pirlimycin, the Committee noted that the analytical method submitted by the sponsor had been validated suitably, however, the mass spectrometry interface was not commercially available anymore and therefore the method would not comply with all Codex requirements for a Regulatory Analytical Method. Since the Committee received information that verification of this method using different equipment was on the way, it recommends that CCRVDF only proposes the MRL for adoption by the Codex Alimentarius Commission if this work has been completed and made available to the WG Methods of Analysis and Sampling in the CCRVDF.

Insecticides

Cyhalothrin

Acceptable daily intake: The Committee established a permanent ADI of 0 – 5 μg/kg bw

Residues definition: Cyhalothrin

Recommended maximum residue limits (MRLs)

Species	Fat (μg/kg)	Kidney (μg/kg)	Liver (μg/kg)	Milk (μg/kg)	Muscle (μg/kg)
Cattle	400	20	20	30	20
Pigs	400	20	20	-	20
Sheep	400	20	50	-	20

Cypermethrin and alpha-cypermethrin

Acceptable daily intake: The Committee established a common ADI of 0–20 μg/kg bw for both cypermethrin and alpha-cypermethrin

Residue definition: Total of cypermethrin residues (resulting from the use of cypermethrin or alpha-cypermethrin as veterinary drugs)

Recommended maximum residue limits (MRLs)

Species	Fat (μg/kg)	Kidney (μg/kg)	Liver (μg/kg)	Milk (μg/kg)	Muscle (μg/kg)
Cattle	1000	50	50	100	50
Sheep	1000	50	50	100	50

Doramectin

Acceptable daily intake: 0-1µg/kg bw (established at the fifty-eighth meeting, WHO TRS 911, 2002)

Residue definition: Doramectin

Recommended maximum residue limit (MRL)

Species	Milk (µg/kg)
Cattle	15[a]

[a] The committee noted that (1) on the basis of a 15 µg/kg MRL for doramectin in whole milk in cattle, the milk discard times would be approximately 240 hours based on the studies using the pour-on treatment. Milk discard times would be approximately 480 hours following treatment using the injection formulated dose; (2) in milk containing 4 per cent milk fat, the residues in milk fat would be equivalent to 375 µg/kg (15 µg/kg ÷ 0.04 = 375µg/kg) This is higher than the 150 µg/kg MRL in fat tissue; (3) the discard time necessary to accommodate the recommended MRL in milk is unlikely to be consistent with good veterinary practice.

Phoxim

Acceptable daily intake: 0 - 4 µg/kg bw (established at the fifty-second meeting (WHO TRS 893, 2000)

Residues: The MRLs for sheep, pigs and goats that were recommended by the fifty eighth (WHO TRS 911, 2002) meeting of the Committee were not reconsidered and maintained.

The temporary MRLs for cattle that were recommended by the fifty- second (WHO TRS 893, 2000) and fifty-eighth (WHO TRS 911, 2002) meeting of the Committee were withdrawn.

Production aids

Melengestrol acetate

Acceptable daily intake: 0-0.03 µg/kg bw (established at the fifty-fourth meeting of the Committee (WHO TRS 900, 2001)

Residues definition: Melengestrol acetate

Recommended maximum residue limits (MRLs)

Species	Fat (µg/kg)	Liver (µg/kg)
Cattle	8	5

Ractopamine

Acceptable daily intake: 0–1 µg/kg bw

Residues definition: Ractopamine

Recommended maximum residue limits (MRLs)

Species	Fat (µg/kg)	Kidney (µg/kg)	Liver (µg/kg)	Muscle (µg/kg)
Cattle	10	90	40	10
Pigs	10	90	40	10

ANNEX 3

GENERAL CONSIDERATION ITEMS

Response to CCRVDF on Draft Risk Assessment Policy

At its 60th meeting the Committee had provided answers to CCRVDF on some specific questions regarding its risk assessment principles (ftp://ftp.fao.org/es/esn/jecfa/ccrvdf60.pdf). On the request of FAO and WHO, the Committee at the present meeting reviewed Annex I of the Discussion Paper on Risk Analysis Principles and Methodologies in the Codex Committee on Residue of Veterinary Drugs in Food (CX/RVDF 01/9 ftp://ftp.fao.org/codex/ccrvdf13/rv01_09e.pdf).

Although the Committee recognised the value of a risk assessment policy, it was concerned that the current draft document to CCRVDF was not adequate due to serious flaws in structure and content.

At the present meeting the Committee agreed that Annex I of the above mentioned draft discussion paper in its current form requires substantial revision, which should consider the following issues:

- A risk assessment policy should provide a general policy framework for the work of risk assessorsand not describe the details of the four steps of the risk assessment process.
- The roles and responsibilities of risk assessors and risk managers need to be clearly defined, recognizing the independence and transparency of the risk assessment process.
- The development of risk assessment guidelines is an inherent part of the corresponding scientific work which needs to be accomplished by risk assessors.
- The Expert Committee is an independent scientific body that provides advice not only to Codex but also directly to FAO and WHO and to member countries. The risk assessment policy needs to recognize these related but independent roles of the Committee.
- The Committee noted that similar activities are on-going in other Codex Committees (e.g. CCFAC, CCFH, CCPR) and therefore strongly recommends that every effort should be made to harmonise these activities.

The Committee recommended that a risk assessment policy (principles and processes) should include at least the following elements:

- Objectives of a risk assessment
- Responsibilities of risk manager and risk assessor in the process of problem formulation
- Need and mechanisms for effective dialogue between risk manager and risk assessor
- Core principles to conduct a risk assessment (e.g. scientific soundness, transparency, etc)
- Inputs to the risk assessment (e.g. sources of data, confidentiality etc)
- Outputs of the risk assessment (form and detail, including request for different risk management options and their consequences)
- Level of protection to be provided by the risk assessment

The Committee welcomed the opportunity to comment on the current document; the Joint Secretariat is asked to continue the discussion with CCRVDF and to consider the possibility of consulting members of JECFA before the next meeting of the Committee in a written procedure. A close co-ordination with other ongoing activities is also desirable.

Conclusions On Specific Toxicological Endpoints

In an effort to improve consistency and transparency, the Committee recommended that a series of standard statements be developed that allow for clear and consistent conclusions on specific toxicological endpoints, in particular on genotoxic and carcinogenic potentials, as well as on reproductive toxicity. The Committee noted that JMPR has developed a set of statements with defined circumstances which should be used as as basis and adapted and/or expanded as appropriate.

The Committee recommended that a small working group, including experts from other JECFA and JMPR panels, should elaborate a set of phrases for conclusions on genotoxic and carcinogenic potentials for discussion at the next meeting, taking into consideration existing efforts. The working group should address standard reporting for other toxicological endpoints as well.

Statistical methods for the estimation of MRLs

On several previous meetings the Committee has discussed that it was desirable to use statistical methods when deriving Maximum Residue Limits for Veterinary Drugs (MRLs) whenever a suitable data base was available. A statistical approach was followed on several occasions where the data met the necessary criteria.

This statistical approach included:

- Linear regression analysis of data describing the terminal depletion of a suitable marker residue in edible tissues following the (last) administration of the drug under approved conditions of use;
- Subsequent use of the results of the regression analysis for the estimation of upper limits of the 95% (alternatively 99%) confidence interval for the upper one-sided tolerance limit on the 95th (alternatively 99th) percentile of the population sampled;
- Iterative calculation of such statistical limits as a function of time over the whole phase of terminal elimination of the marker residue;
- The statistical method includes a mechanism for the derivation of Maximum Residue Limits for Veterinary Drugs from a set of data.

Since the necessary calculations are complex and should be performed reproducibly and in a fully transparent manner, the Secretariat has supported the development of a tool which is based on spreadsheets and which facilitates the application of the necessary statistical tests to kinetic residue depletion data and the calculation of the above mentioned statistical tolerance limits. The currently available test version supports the estimation of suitable MRLs for edible tissues. The workbook uses only basic EXCEL instructions. Intentionally no use of sophisticated programming has been made in order to allow the user to control every individual calculation and fully understand the procedure.

The Committee welcomed the initiative of the Secretariat and recommended that the Secretariat continues with the necessary steps:

- to further improve the current applications and the documentation of the tool;
- to extend the applicability of the tool to include estimation of MRLs for milk;
- to publish the tool and invite all interested parties to comment on it;
- to test and validate the tool.

Lipid Soluble Residues of Veterinary Drugs with MRLs in Milk

At this meeting of the Committee, consideration was given to the potential public health impact of lipid soluble residues of veterinary drugs in milk where milk fat may be used for production of processed dairy products. Examples of classes of particular compounds include, but are not necessarily limited to, the macrocyclic lactones and pyrethroids.

The Committee has routinely tried to harmonize its recommendations on MRLs where possible with the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) and the Codex Committee on Pesticide Residues (CCPR), particularly in those situations where a substance may be used as a pesticide or as a veterinary drug. For a substance such as the cypermethrins, for example, JMPR recommends MRLs in animal milk based on milk fat content. In this regard, reporting a MRL of a lipid soluble compound in cattle milk on a milk fat basis would be consistent with JMPR procedures. Further, this would permit consideration for a single MRL for a substance regardless of its origin either as a veterinary drug or as a pesticide.

At previous meetings of the Committee where MRLs for these classes of compounds have been considered, the Committee has limited its MRL recommendations to fresh milk rather than including recommendations as MRLs in milk fat where large concentration factors occur. This is consistent with the definition of an MRL in raw, unprocessed products. However, the definition does take into account other relevant risks as well as food technological aspects. An example of the effect of reporting an MRL on a milk fat basis is demonstrated by a situation with an MRL of 1 mg/kg in whole milk. If fresh milk contains four percent milk fat, the MRL value in milk fat would be 25mg/kg (1 mg/kg ÷ 0.04 = 25mg/kg), assuming all residue partitions into the milk fat.

In those situations where milk or milk fat may be used in producing commodities such as butter and cheese, milk fat may be a very high percentage of the finished product and result in very high amounts residues. These highly elevated amounts of residues in the finished, processed product may cause public health concerns, if resulting in amounts of residues that may exhibit an effect in humans. Such determination would have to be considered on a case by case basis.

Recognizing the potential public health consequences identified by this matter, the Committee requests early consideration by the Codex Committee on Residues of Veterinary Drugs (CCRVDF) as risk managers on how JECFA should proceed in the future where MRLs of lipid soluble residues in milk originating from the use of veterinary drugs are identified. It should be noted that if CCRVDF indicates the Committee to proceed in this manner, it would require JECFA to reconsider those MRLs where lipid soluble residues with MRLs in whole milk have been recommended.

Analytical Terminology for Codex Use

The Committee considered a document on proposed revised definitions of analytical terminology contained in the Codex Procedural Manual prepared by the Codex Committee on Methods of Analysis and Sampling, CCMAS (CL 2003/43-MAS). It noted that the Committee report, FAO Food & Nutrition Paper 41/14 contains a section on Requirements for Validation of Analytical Methods. The CCMAS document generally references Codex definitions and provides guidance on the experimental data required response to the definitions. Several proposed revised definitions, however, are of analytical terms also defined in the FAO Food & Nutrition Paper 41/14. The Committee was also aware that the Codex Committee on Residues of Veterinary Drugs in Foods is reviewing requirements for analytical methods, residues of veterinary drugs in foods. The Committee agreed in principle that definitions of analytical terminology used in JECFA documents should be harmonized with definitions used in the Codex Procedural Manual and in Codex Volume 3.

Since work is in progress in the Codex Committees and final definitions have not been approved by Codex Alimentarius Commission, the 62nd JECFA Committee agreed that this matter should be considered at the next meeting of the Committee. It recommended that an expert should be assigned to review and report on the status at that Meeting.

ANNEX 4

List of compounds which have been evaluated by JECFA but for which an ADI and/or MRL was not recommended *)

Veterinary drug	JECFA	Year	Explanation (and reference)
Bacitracin 1)	12	1968	If bacitracin is used, it should not be allowed to give rise to detectable residues in food for consumption by humans. If the methods of analysis recommended by the Committee) are used, it will be possible to ensure that the residues in food will not exceed the following limits: milk, 0-1.2 IU/ml ; meat, 0-0.7 IU/g ; and eggs, 0-4.8 IU/g (1 mg of bacitracin = 42 IU).
Carbadox	60	2003	The new data confirm that carcinogenic residues, in particular desoxycarbadox, are present in edible tissues during the depletion of parent carbadox. The relatively long persistence of the residues was a new finding. The results also show that, after administration of the highest recommended dose of 55 mg/kg in feed, QCA depletes to below the MRL for liver recommended by the Committee at its 36th meeting within a short time (approximately 17 days on the basis of the upper limit of the 95% confidence interval on the 99th percentile). [...] As the Committee was unable to allocate an ADI for carbadox, there is no accepted reference point for comparison with the new data on residues. Therefore, on the basis of the new data, the MRL for QCA recommended by the Committee at its thirty-sixth meeting is not supported for determining residues of carbadox of toxicological concern in liver. The MRL of 5 µg/kg recommended by the Committee at its thirty-sixth meeting for QCA in muscle is not supported by the new data. Desoxycarbadox was found at all times up to 15 days, but QCA was found in only two samples collected 0 and 3 h after withdrawal. Therefore, the relationship between the concentrations of QCA and desoxycarbadox is not known. After reviewing the new studies, the Committee could not determine the amounts of residues of carbodox in food that would have no adverse health effects in consumers. The Committee decided to withdraw the MRLs of carbadox recommended by the Committee at its 36th meeting. FNP 41/15
Cefuroxime	62	2004	After consideration of all available data, including additional residue information provided to the Committee and considering that: - No new information had been provided in response to requests for data on the identification and toxicity of the unidentified residues of cefuroxime in milk; - The Committee was unable to adequately evaluate cefuroxime metabolism or degradation in milk; and - The radiolabelled-residue depletion study in cows can no longer be used to determine the relationship between residues of parent compound, other antimicrobial active residues and total residues of cefuroxime. The present Committee concluded that it could not extend the temporary ADI or MRLs established at the fifty-eighth meeting. Therefore, the temporary ADI and MRLs for cefuroxime in milk were withdrawn.
Chloramphenicol	42	1994	The Committee was unable to establish an ADI for chloramphenicol both because of the lack of the information needed to assess its carcinogenicity and effects on reproduction, and because the compound was genotoxic in a number of in vitro and in vivo test systems. The Committee was unable to assign MRLs for chloramphenicol primarily because no ADI was allocated. In addition, insufficient information was available to identify a suitable marker residue, particularly in cattle and pigs, for which radiodepletion studies were inadequate. TRS 851

*) This list was prepared by the FAO Joint Secretariat based on the on-line edition of FAO Food and Nutrition paper 41 (http://www.fao.org/es/esn/jecfa/jecfa_vetdrug_en.jsp). As requested by CCRVDF, the database and the list contains short explanations of the reasons why ADI/MRLs had not been established. It shoud be noted that this list is for information only and does not substitute the official reports and other publications from JECFA.

Veterinary drug	JECFA	Year	Explanation (and reference)
Chlorpromazine	38	1991	In view of the lack of relevant toxicological data, the long-term persistence of chlorpromazine in humans, the spectrum of additional effects of the drug, and the probability that even small doses can cause behavioural change, the Committee was unable to establish an ADI. Furthermore, the Committee suggested that chlorpromazine should not be used in food-producing animals. TRS 815
Dexamethasone	50	1998	The Committee concluded that the analytical method did not meet the required performance criteria for the identification and quantification of incurred residues of dexamethasone in tissues. Therefore, the method was not considered to be suitable for the analysis of dexamethasone residues for regulatory purposes. In the absence of an acceptable analytical method for monitoring purposes, the Committee was unable to recommend MRLs for dexamethasone. TRS 888
Diethylstilboestrol	5	1960	The compound was considered at the 5th meeting which was convened by FAO/WHO to assess the problem of possible carcinogenic action of food additives.
Dimetridazole	34	1989	Although a no-observed-effect level of 100mg/kg in the diet, equal to 4mg per kg of body weight per day, was reported in the multidose long-term rat study, the Committee could not establish an AD1 solely on the basis of this study in the absence of the results of a carcinogenicity study in a second species. TRS 788
Enrofloxacin	48	1997	The substance received at the 48th meeting a full ADI but the evaluation of the residue data due to the late submission of important additional data was postponed.
Erythromycin 1)	12	1968	Acceptable levels of residues in food: If antibiotics of this group are used, they should not be allowed to give rise to detectable residues in human food. If the methods of analysis recommended by the Committee are used, it will be possible to ensure that the residue levels in food for human consumption will be within the following limits (ppm) :Milk 0-0.4, Meat 0-0.3, Eggs 0.0.3. TRS 430
Furazolidone	40	1992	The Committee did not recommend an MRL, because: - no ADI was established; - the residue data presented to the Committee were not sufficient for it to identlfy a marker residue; and - insufficient information was available on the quantity and nature of the total residues. TRS 832
Ipronidazole	34	1989	The Committee was not able to establish an ADI because the rat carcinogenicity study was inadequate to determine a no-effect level for ipronidazole. TRS 788
Kanamycin 1)	12	1968	Kanamycin could not be fully evaluated toxicologically on the basis of the data that were available. TRS 430
Leucomycin 1)	12	1968	Additional data are required before leucomycin can be fully evalu- ated. The additional biological information should include the results of adequate toxicity studies and studies of bacterial resistance and cross resistance with other macrolide antibiotics. TRS 430
Metronidazole	34	1989	Metronidazole was not evaluated toxicologically because the relevant data were not made available to the Committee. The depletion of residues of metronidazole in food-producing animals has not been studied. TRS 788

Veterinary drug	JECFA	Year	Explanation (and reference)
Nitrofural	40	1992	The Committee did not recommend an MRL, because: - no ADI was established; - the residue data available to the Committee were not sufficient for it to identify a marker residue; and - no information was available on the quantity and nature of the total residues. TRS 832
Nitrofurazone	40	1992	The Committee did not recommend an MRL, because: -no ADI was established; - the residue data available to the Committee were not sufficient for it to identify a marker residue; and - no information was available on the quantity and nature of the total residues. TRS 832
Novobiocin 1)	12	1968	Acceptable levels of residues in food When novobiocin is used, it should not be allowed to give rise to detectable residues in food for human consumption. Use of the methods recommended by the Committee will make it possible to ensure that the residues in food will not exceed the following limits (ppm) : milk, 0-0.15; meat, 0-0.5; and eggs, 0-0.1. TRS 430
Nystatin 1)	12	1968	Since nystatin is used only externally and not in food itself, only trace amounts are likely to be present in food for human consumption. If the methods of analysis recommended by the Committee are used, it will be possible to ensure that the residue levels in such food will not exceed the following levels (ppm) : milk, 0-1.1 ; meat, 0-7.1 ; and eggs, 0-4.3. TRS 430
Oleandomycin 1)	12	1968	Acceptable levels of residues in food If antibiotics of this group are used. they should not be allowed to give rise to detectable residues in human food. If the methods of analysis recommended by the Committee are used, it will be possible to ensure that the residue levels in food for human consumption will be within the following limits (ppm) : Milk: 0-0.15, Meat: 0-0.3, Eggs: 0-0.1. TRS 430
Oxolinic Acid	43	1994	In view of the major deficiencies in the reporting and protocols of the toxicological studies available for evaluation, and as a clear NOEL in the arthropathy study in dogs could not be identified, the Committee was unable to establish an ADI. (...) The Committee was not able to set MRLs for oxolinic acid because no ADI was established. No additional residue data were requested. TRS 855
Permethrin	54	2000	An ADI of 0-50 mg/kg bw for technical grade permethrin with cis:trans ratios of 25:75 to 40:60 was established by the 1999 Joint FAO/WHO Meeting on Pesticide Residues (JMPR; FAO Plant Production and Protection Paper 153, Rome, 2000). At its fifty-forth meeting (2000), the Committee was unable to establish an ADI for the 80:20 cis:trans isomeric mixture proposed for use as a veterinary drug because of the lack of information on toxicity. In the absence of an ADI, the Committee was unable to recommend MRLs for the 80:20 cis:trans isomeric mixture of permethrin.
Polymixin B 1)	12	1968	Acceptable levels of residues in food If polymyxin B is used, it should not be allowed to give rise to detectable residues in human food. If the methods of analysis recommended by the Committee are used, it will be possible to ensure that the residues in food will not exceed the following limits : milk, 0-2 IU/ml ; meat, 0-5 IU/g ; eggs, 0-5 IU/g. Recommendations (1) Polymyxin B should be considered acceptable as at present used. (2) Long-term studies of the effects of low residue levels should be carried out and the results submitted to WHO within 5 years. TRS 430

Veterinary drug	JECFA	Year	Explanation (and reference)
Propionylpromazine	38	1991	The Committee was not able to set an MRL because: - no ADI was established; - the residue data were insufficient, in that no depletion study was carried out, and no marker residue or target tissue was identifiable. The Committee also expressed concern about the high levels of residues at the injection site. The Committee was unable to recommend the continued use of propionylpromazine in food-producing animals and, before it would consider the compound again, would require a full range of toxicological and residue data, including data from which a dose producing no pharmacological effects in humans could be established. TRS 815
Ronidazole	42	1994	Ronidazole had previously been evaluated at the thirty-fourth meeting of the Committee, when a temporary ADI of 0-0.025 mg per kg of body weight was established. Additional data were required for consideration by the Committee. New data were not made available to the Committee at the present meeting, and the temporary AD1 was therefore not extended. TRS 851
Sulphthiazole	34	1989	The Committee did not establish an ADI because of the lack of data on the hormonal effects of sulfathiazole. (...) To be able to assess the drug properly, the Committee considered that adequate residue and radiometric studies were needed. TRS 788
Thiamphenicol	58	2002	The Committee at its fifty-second meeting established an ADI of 0–5 mg/kg bw on the basis of a microbiological end-point. In addition, the temporary MRLs for poultry and cattle were withdrawn because the data submitted only partly addressed the Committee's request at its forty-seventh meeting. Temporary MRLs were recommended for pig, of 50 µg/kg in muscle and fat, 100 µg/kg in liver and 500 µg/kg in kidney, and for fish, of 50 µg/kg in muscle with adhering skin. The MRLs were designated as temporary, pending the results of a study with radiolabelled drug in pigs to determine the relationships between total residues and free and conjugated thiamphenicol in all tissues, and a validated analytical method for tissues from all animal species which includes an enzymatic hydrolysis step to allow determination of the sum of thiamphenicol and thiamphenicol conjugates as free thiamphenicol. As no information was submitted for consideration at the present meeting, the Committee did not extend the temporary MRLs. TRS 911
Tylosin	38	1991	Because of the deficiencies in the toxicological and microbiological data, the Committee was not able to establish an ADI. (...) The Committee was not able to set an MRL because no ADI was established. TRS 815
Xylazine	47	1996	The Committee did not recommend MRLs for xylazine because: - no ADI was established; - the data on the metabolism of the compound were inadequate; - the residue data available to the Committee were not sufficient for it to identify a marker residue; - the residue-depletion studies were inadequate. TRS 876

Note 1): This compound was not re-evaluated after the program for the risk assessment of residues of veterinary drugs in foods of animal origin started in 1987 (32nd meeting).