RACTOPAMINE HYDROCHLORIDE

First draft prepared by

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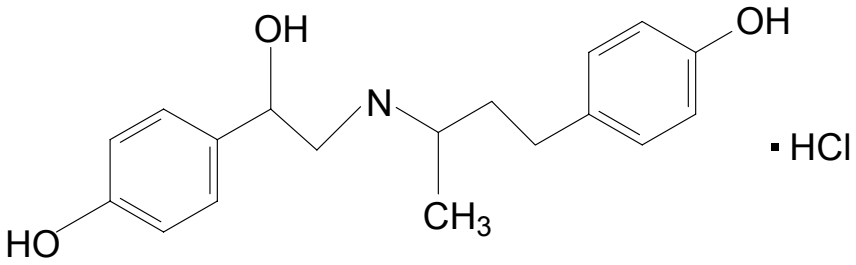
IDENTITY

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

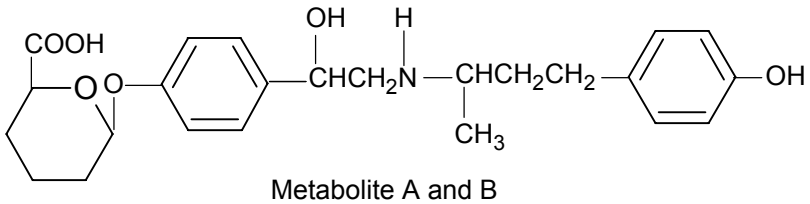
Laboratory Animals

Rats

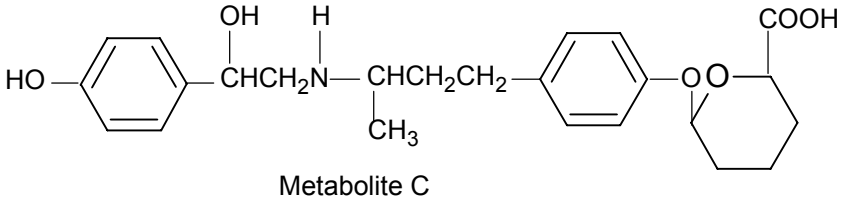
All studies in rats were performed using ^{14}C labeled ractopamine in which the radiolabel was attached in the hydroxyphenylethyl ring (ring "A") or the hydroxyphenylbutyl ring (ring "B") of ractopamine. The two differently radiolabeled ractopamines were then combined and used in radiochemically equivalent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

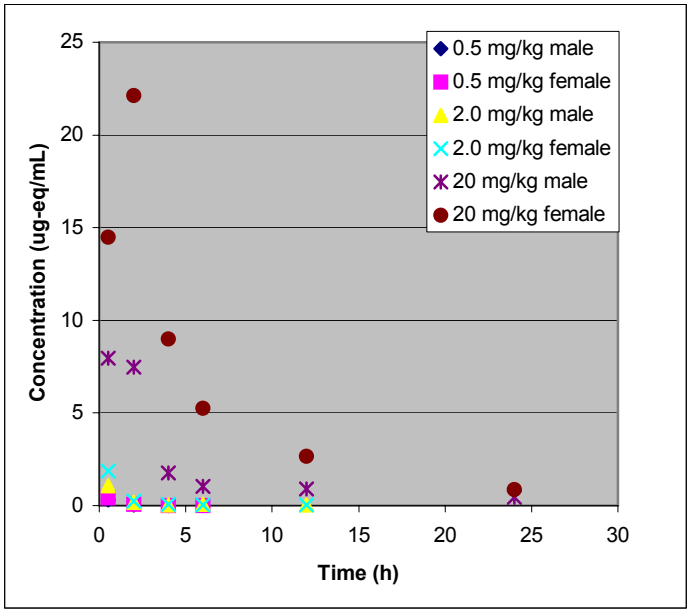
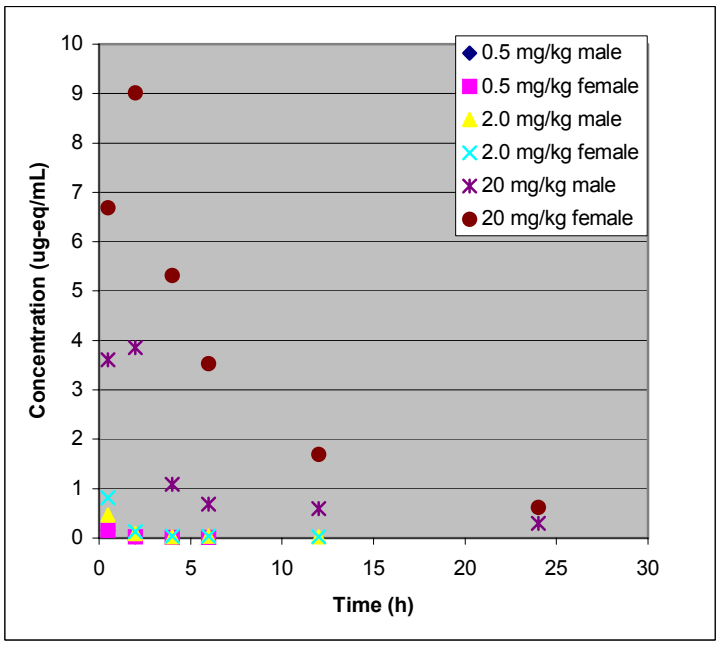


Figure 2b Concentration of ^{14}C-ractopamine hydrochloride (as µg-eq/mL) in blood after single dose administration by oral gavage at 0.5, 2.0, and 20 mg/kg to rats.



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.

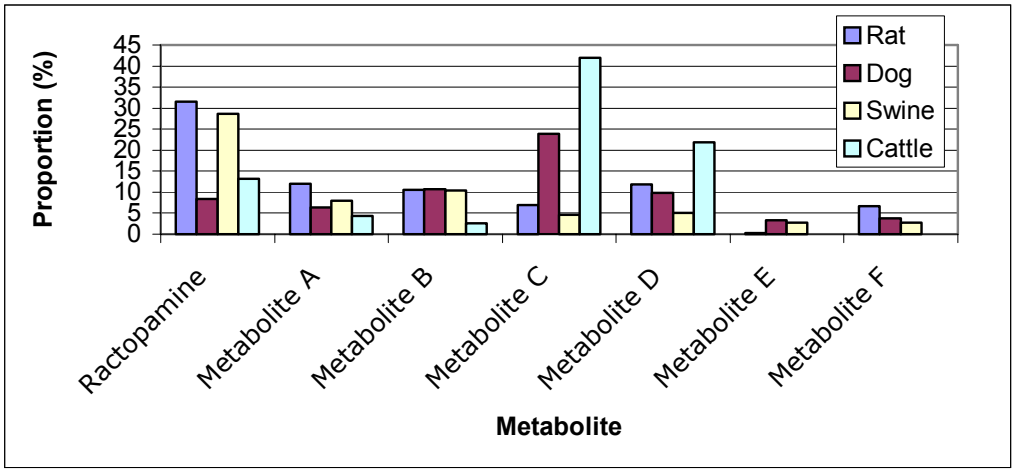
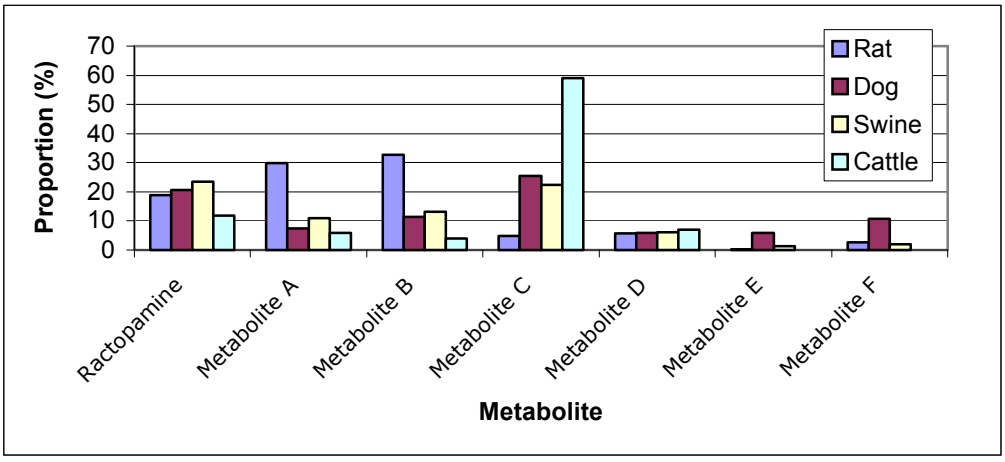


Figure 3b Poportional composition of ractopamine and it's metabolites A, B, C, D, E, F in rat, dog, swine and cattle kidneys.



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.

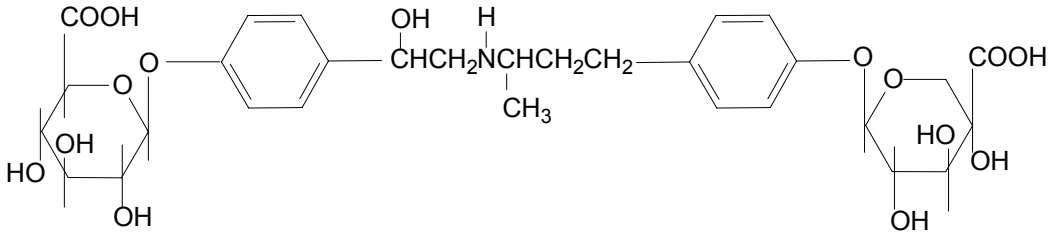
In another GLP study, 6 cross-bred pigs (each approx. 45 kg bw) received 30 mg/kg of ^{14}C-ractopamine hydrochloride in a special ration for 4 days, then killed 12 hours after the last dose (Dalidowicz, 1987b). Parent ractopamine accounted for 28.7% (0.12 mg/kg) and 23.4% (0.10 mg/kg) of the total extractable 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.

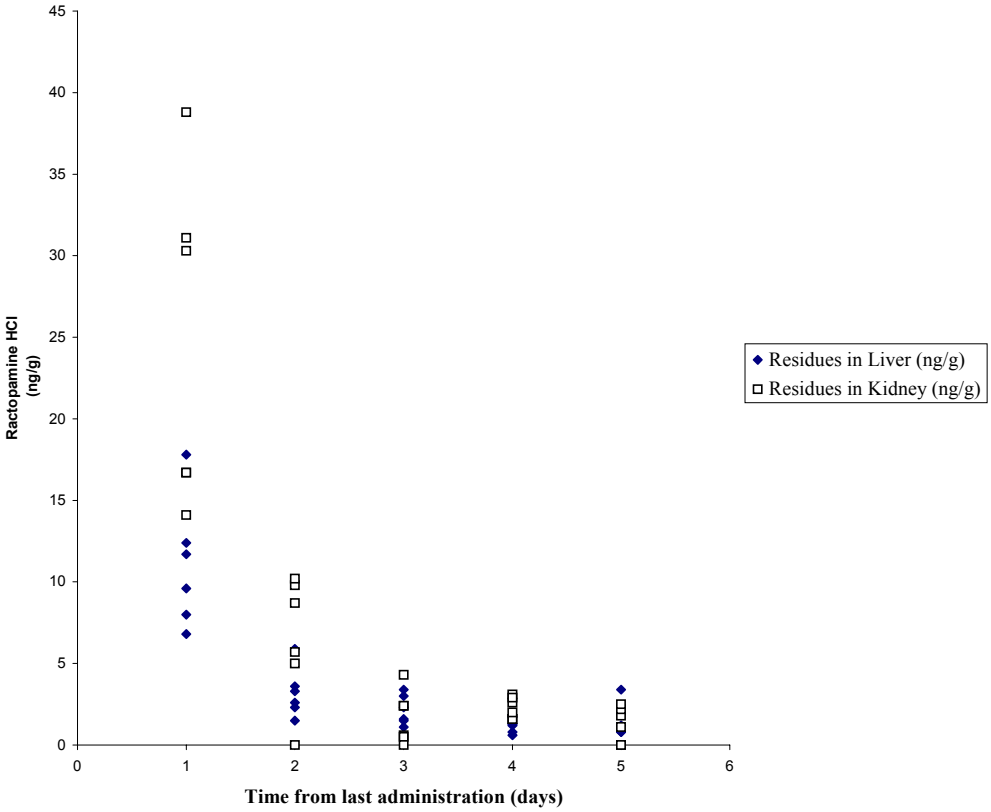
Table 4 Residues of ractopamine (parent compound) in tissues from pigs which received a ration containing either 10 mg/kg or 15 mg/kg of the drug in feed for 6 days.

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

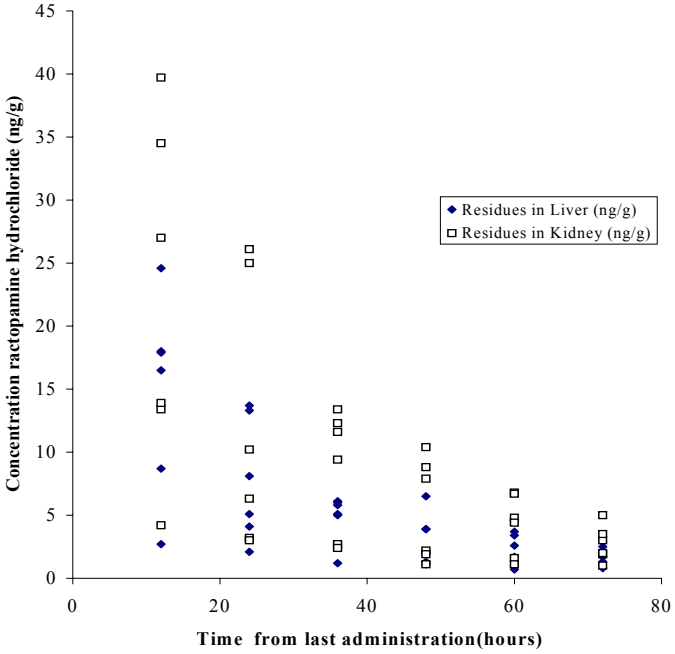


Table 5 Depletion of ractopamine residues in pigs fed a ration containing 20 mg/kg ractopamine hydrochloride for 14 days.

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).

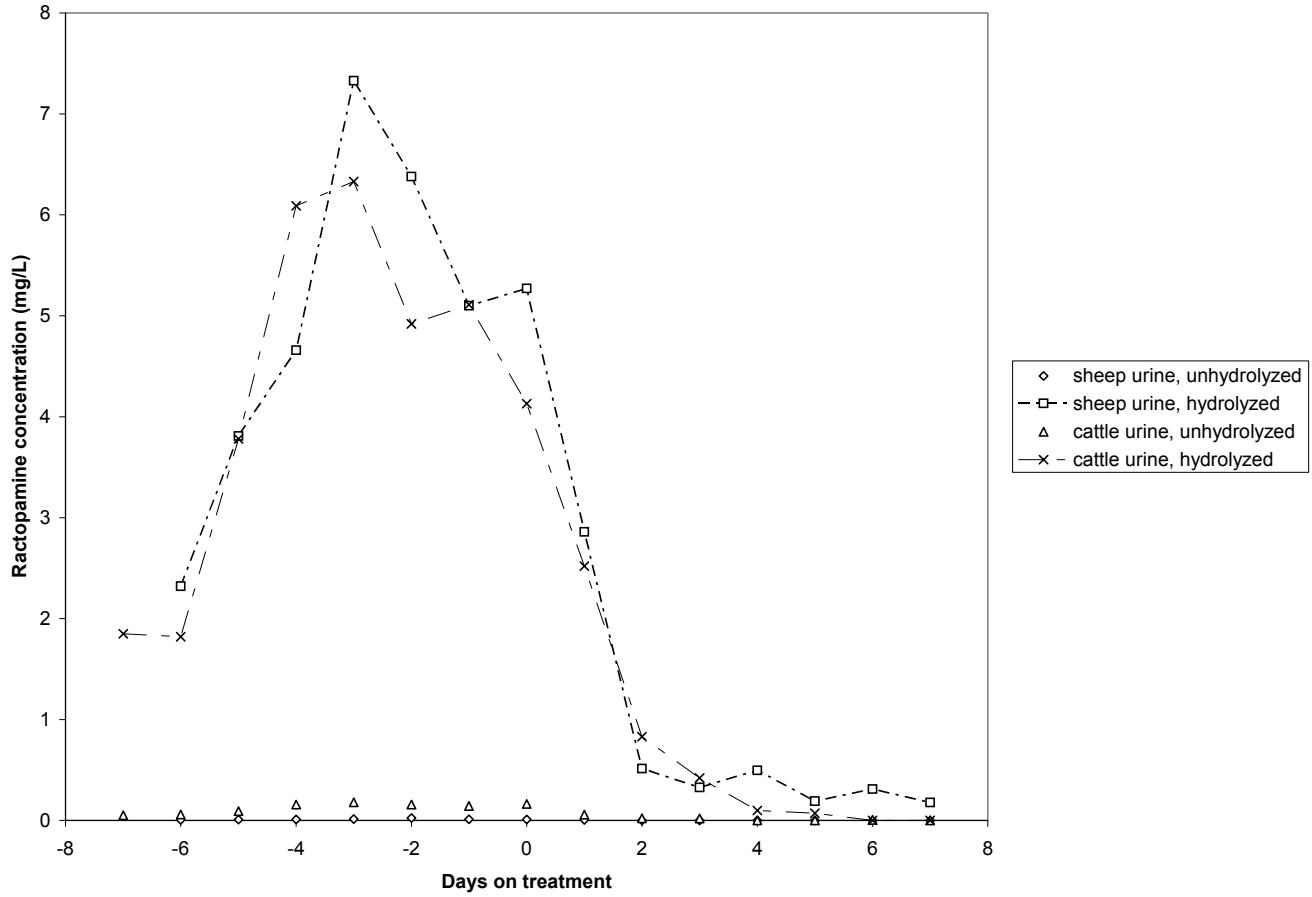
Cattle and other species

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.

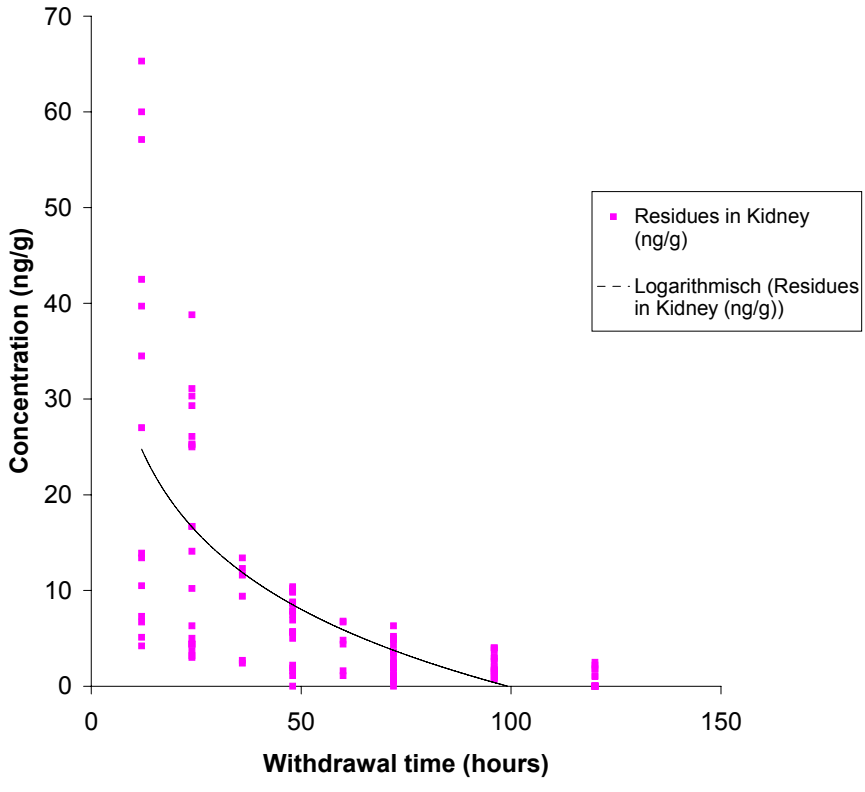
More recently, a method has been published for the detection of ractopamine residues in pork and beef muscle, using LC-fluorescence and LC/MS/MS (Shishani et al, 2003). This method includes an initial extraction with methanol, incubation with β-glucuronidase (*H. pomatia*) at 65oC for 2 hr, then extraction 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

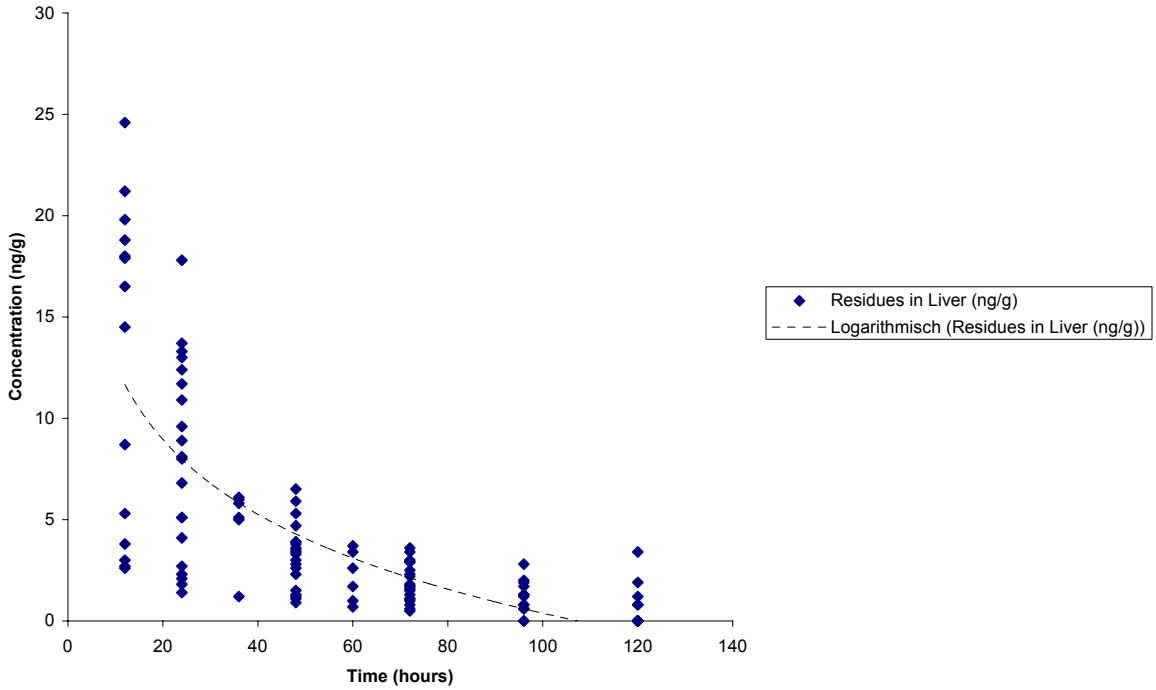
chromatography with electrochemical detection and later studies used fluorescence detection, which has been proposed as the regulatory method.

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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