Proceedings of the 15th International Conference of Racing Analysts and Veterinarians, Dubai, United Arab Emirates

METABOLISM OF THE BETA-ADRENERGIC AGONIST RACTOPAMINE IN HORSES AND CONFIRMATION OF ITS ADMINISTRATION WITH GC/MS

A. F. Lehner, C. G. Hughes, J. D. Harkins, J. Bosken, F. Camargo, J. Boyles, A. Troppmann, W. Karpiesiuk, B. Mollett, W. E. Woods and T. Tobin

Maxwell H. Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky, Lexington, Kentucky 40546-0099, USA

ABSTRACT

The metabolism and detection of the beta-adrenergic agonist ractopamine was investigated in the horse. Ractopamine is typically administered to livestock as Paylean® with the intention of altering muscle lean-to-fat ratios. It is recognised by the Association of Racing Commissioners International (ARCI) as a Class 2 foreign substance, indicating a significant potential to affect performance in racehorses. Urinary metabolites of ractopamine were identified by direct infusion ESI^+ MS/MS as glucuronide, methyl, mixed methyl-glucuronide, sulphate and mixed methyl-sulphate conjugates. We also considered the theoretical possibility that a previously unrecognised conjugate added 113 amu to produce m/z 415 [M+H] species, or 2 x 113 amu to give m/z 528 [M+H] species, either through covalent amino acid conjugation, or through non-covalent creatinine complexation.

Screening of urine with an ELISA kit that provided a linear response between 1.0–100 ng/ml and an effective limit of detection of 50 ng/ml readily detected ractopamine equivalents in urine from Paylean®-dosed horses. Ractopamine equivalents could be detected up to 24 h after a 300 mg oral dose. Enzyme hydrolysis, solid phase extraction (SPE) and TMS derivatisation enabled GC/MS confirmation with selected ion monitoring (SIM) of ractopamine-tris(TMS) ions, in comparison to an isoxsuprine-bis(TMS) internal standard. The instrumental limit of detection was measured as 0.1 ng, corresponding to roughly 5 ng/ml in matrix. Standard curves demonstrated a second-order curvilinear response for ractopamine between 10 and 1,000 ng/ml with a correlation coefficient r >0.99. The lower limit of detection for ractopamine in urine at which diagnostic MS ion ratios confirmed ractopamine parent drug was 25–50 ng/ml. GC/MS quantified ractopamine in urine at >350 ng/ml 24 h post dose following 300 mg orally. Overall, the results indicate that detection and confirmation of ractopamine in equine urine can be accomplished readily with ELISA-screening followed by GC/MS confirmation.

INTRODUCTION

Ractopamine, N-[2-(4-hydroxyphenyl)-2-hydroxyethyl]-1-methyl-3-(4-hydroxyphenyl) propylamine, is a beta-adrenergic agonist marketed as Paylean® and approved for use in swine and other livestock as a growth regulator. It has a ß-hydroxy-phenethylamine structure common to many beta-adrenergic agonists (Fig 1), and has the property of altering muscle lean-to-fat ratios. This results in reduction of fat, increased muscle mass and improved feed utilisation efficiency in swine, cattle and turkeys (Smith 1998). Ractopamine promotes protein deposition with little effect on fat deposition in the pig.

Ractopamine treatment results in a stimulation of myofibrillar protein synthesis and elevates absolute rates of protein synthesis and breakdown in biceps femoris muscle in the pig. The result is enhancement of protein accretion in skeletal muscle of pigs (Helferich *et al.* 1990). In rats, ractopamine increases methionine incorporation in cultured muscle cells (Anderson *et al.* 1990). Studies in rats indicate that of the 4 possible

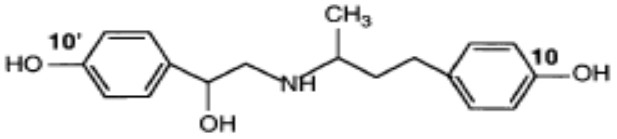

Fig 1: Two-dimensional structure of the beta-adrenergic agonist ractopamine, indicating the location of position 10 and 10' phenolic hydroxyls.

ractopamine stereoisomers (RR, RS, SR, SS), the RR isomer is responsible for the majority of leanness-enhancing effects (Ricke *et al*. 1999).

Both conjugated and non-conjugated metabolites of ractopamine have been reported in various species. In cattle and sheep, for example, ractopamine residues in urine samples were measured before and after hydrolysis of conjugates. The data indicate that after the hydrolysis of conjugates, ractopamine should be detectable in urine of sheep for as long as 7 days after the last exposure and as long as 5 days after withdrawal in cattle (Smith and Shelver 2002).

Conjugate metabolite structures have been identified in rat bile as a sulphate-ester/glucuronic acid diconjugate of ractopamine. The site of sulphation was at the C-10′ phenol (phenol attached to carbinol) and glucuronidation was at the C-10 phenol (phenol attached to methylpropylamine) of ractopamine (Smith *et al*. 1995; cf. Fig 1).

Enzyme immunoassay-based detection of ractopamine has been made possible through commercial ELISA kits. Shelver and Smith (2002) demonstrated applicability of a monoclonal antibody-based ELISA for determination of ractopamine residues in sheep and cattle samples.

Ractopamine is an ARCI Class 3 drug but not a recognised therapeutic medication in racehorses. It is a candidate for abuse due to its potential to affect performance. Therefore, improper use needs to be monitored with reliable analytical methodology, as its discovery in performance horses may lead to sanctions against owners or trainers.

This study aimed to determine the metabolism of ractopamine in the horse and elucidate the structure(s) of target metabolites for mass spectrometric confirmation of its administration. HPLC methods with various detectors exist for ractopamine (Turberg *et al*. 1996; Smith and Shelver 2002), although electrospray ionisation mass spectrometry is rapidly becoming more widespread with a proliferation of methodologies (Antignac *et al*. 2002; Churchwell *et al*. 2002). In addition to mass spectrometric analyses, we determined detectability by ELISA, commercial kits of which are widely available for screening purposes in racing chemistry laboratories.

Materials and methods

Three mature Thoroughbred mares (about 550 kg) were placed in stalls 24 h prior to experimentation. They were fed twice a day with grass hay and feed (12%), which was a 50:50 mixture of oats and an alfalfa-based protein pellet. They had been vaccinated annually for tetanus and de-wormed quarterly with ivermectin (MSD Agvet, New Jersey, USA). A routine clinical examination was performed before each experiment to assure they were healthy and sound. During experiments, horses had water and hay *ad libitum*. Each mare served as its own control. The animals were managed according to the regulations of the University's Institutional Animal Care Use Committee, which also approved the experimental protocol. Ractopamine was administered orally, as a feed supplement Paylean® (Elanco, Charlotte, NC). Doses were 300 mg in 85 g Paylean® for ELISA and GC/MS analysis and 900 mg in 255 g Paylean® for detailed metabolism studies. Urine was collected immediately before and at 1, 2, 4, 6, 8 and 24 h after administration using a Harris flush tube (24 Fr diameter x 60 in; Seamless, Florida, USA). Urine samples were divided into aliquots and stored at -20°C until assayed.

Sample collection and preparation

The ractopamine standard for mass spectral analysis was obtained from the USDA as ractopamine-HCl. ESI(+)-MS/MS showed it to consist of ractopamine with no apparent contaminants. Pre- and post administration urine samples were treated by solid phase extraction (SPE). The filtrate was then diluted 1:10 with a mixture of 50:50 acetonitrile and 0.05% formic acid (aq) for positive mode mass spectrometry (ESI(+)-MS/MS). The mixture was infused 1.2 ml/h via a Harvard syringe pump equipped with a 500 µl Hamilton gas-tight syringe. Infusion was direct into the electrospray probe of the Quattro II MS/MS (Micromass, Massachusetts, USA).

ELISA detection

Testing Components Corp (Illinois, USA) ractopamine ELISA kits were used to establish the detection limit for ractopamine in equine urine. The assay was performed according to manufacturer's instructions: A stock solution (1 mg/ml) of ractopamine was prepared in methanol. ELISA standards (0.1 to 1,000 ng/ml) were prepared by dilution of stock with ELISA kit assay buffer. The ELISA kit consisted of microtitre plates, antibody #1, antibody #2 (goat anti-rabbit) concentrate, assay buffer (phosphate buffered saline, pH 7.4), wash solution 20X concentrate and substrate. The assay was started by pipetting 50 µl standard solutions samples into the appropriate wells. Antibody #1 solution (100 µl) was added to each well followed by gentle mixing and incubation for 30 min at 37°C. The plate was then washed 3 times with diluted wash solution (300 µl). Freshly diluted antibody #2 (150 µl) was added to each well with gentle mixing. The plate was incubated 30 min at 37°C. The plate

was again washed 3 times as above. Substrate (100 µl) was added to each well, and the optical density was read at 650 nm with an ELX800 Microplate Reader (BioTek Instruments, Inc., Vermont, USA) 15 min after initiating the incubation.

Beta-glucuronidase hydrolysis

Urine samples were treated for 3 h at 65°C with *Patella vulgata* beta-glucuronidase (1,000 units of Sigma Type L-II/ml urine brought to 0.25 M sodium acetate, pH 5). The resultant hydrolysates were subjected to SPE as described below.

Solid phase extraction and derivatisation

SPEs were run on a Speedisk 48 Pressure Processor (SPEware Corp., California, USA). SPE columns (United Chemical Technologies, Pennsylvania, USA, type CSDAU Clean-screen) were conditioned by adding sequentially 3 ml methanol, 3 ml water and 1 ml 0.1M sodium phosphate buffer, pH 6.0. Samples were loaded and the column washed sequentially with 2 ml water, 2 ml 1M acetic acid, 4 ml methanol. The column was dried with N_2 (20 psi nominal pressure) for 1 min. Then the column was eluted with 3 ml dichloromethane/isopropanol/NH_4OH (concentrated) (78:20:2, v:v:v) into glass tubes. The eluent was evaporated to dryness under a stream of N_2 in a 35–40°C water bath. The residue was dissolved by vortexing in 15 µl of N,N-dimethylformamide and 50 µl of BSTFA-1% TMCS (Pierce Chemicals, Illinois, USA), then transferred to a micro-injection vial and sealed. Derivatisation occurred upon dissolution and/or in the 250°C injector port; incubation at 70°C for 30 min prior to injection did not increase the yield of derivative: 1 µl was injected into the GC/MS. When ESI(+)MS analysis was the preferred method, re-suspension of dried eluents took place directly in 1 ml acetonitrile:0.05% formic acid, 1:1.

GC/MS SIM confirmation

Confirmation of ractopamine in urine following beta-glucuronidase hydrolysis, SPE extraction and TMS derivatisation was achieved by GC/MS SIM. The GC column was a HP-5 MS, 30 m × 0.25 mm × 0.25 µm film thickness in the splitless mode with 1 ml/min helium. The GC oven temperature programmed was: 180°C for 2 min, increased to 280°C at 20°C/min, held at 280°C for 10 min. Data were collected following a 1 µl injection as follows. Isoxsuprine was used as an internal standard due to its similar structure and its uniform, predictable behaviour during GC/MS analysis (Bosken *et al.* 2004). The m/z 178 base peak of isoxsuprine-bis(TMS) was monitored for quantitation. Ions monitored for the tris(TMS)-ractopamine derivative were m/z 267 [quantitative ion], 250, 179 and 502, in order of decreasing abundance. Preparation of the ractopamine standard curve was accomplished by determining the internal standard (m/z 178) and ractopamine (m/z 267) peak areas for a series of standards (0, 10, 20, 50, 100, 200, 300, 500, 1,000 ng/ml), and plotting the calculated ratio of ractopamine area/internal standard area on the horizontal axis versus concentrations on the vertical axis of a 2-d plot. Standard curves prepared this way gave coefficients of determination R^2>0.99. For GC/MS scanning experiments, the m/z 50–700 mass range was scanned at 1.19 scans/s.

MS/MS analysis

Full scan electrospray ionisation (ESI) mass spectra were obtained on analytical standards at 10 µg/ml in 50:50 acetonitrile: 0.05% formic acid (aq), pH~4, by infusion at 1.2 ml/h via a Harvard syringe pump into the electrospray probe of a Micromass Quattro II MS/MS set in positive ion mode. All spectra were optimised by combination of 1–2 min of uniformly acquired data, background subtraction and peak smoothing.

MS/MS tuning

The mass spectrometer was tuned for positive ion spectra by direct infusion of 10 ng/µl ractopamine in 50:50 acetonitrile: 0.05% formic acid (aq). Peak shape and intensity of the monoprotonated ractopamine m/z 302 ion were optimised by adjustment of capillary, HV lens, cone voltage, skimmer lens and RF lens settings. Skimmer lens offset was left at 5 V. Collision gas (argon) and collision energy were adjusted for collisionally-induced dissociation (CID) in the central hexapole by optimising settings as needed for the second quadrupole. Generally, the collision gas was set to 1–3 × 10^{-3} mbar. Increasing the photomultiplier setting 100–150 V above the regular 650 V sufficiently increased sensitivity. In general, for positive mode, the source cone voltage was set at 24 V, the collision energy at –20 V, the capillary of the ESI probe at +3.0 kV, the skimmer at 2.1 V and the HV lens at 0.54 kV. Source temperature was 120°C.

RESULTS

Ractopamine by ESI(+) MS

Ractopamine, 301 mw, readily produced a M+H pseudomolecular ion at m/z 302 when dissolved

in acetonitrile:0.05% formic acid and examined by ESI(+)MS. The product ion spectrum of this m/z 302 species gave intense responses particularly at m/z 107, 121, 136 and 164. Urine collected at 4 h post dose (900 mg) was subjected to beta-glucuronidase treatment. The product ion spectrum of the SPE m/z 302 material from the dosed horse is an excellent match to that of the authentic ractopamine. The product ion spectrum is also a match for that of a pronounced m/z 302 peak in the commercial formulation Paylean, verifying that no prodrugs or unintended conjugates were present in the feed version.

Ractopamine metabolites by ESI(+)MS

ESI(+) mass spectrometry revealed metabolites of ractopamine in urine by comparison of glucuronidase-treated SPE extractions of pre-dose urine (0 h) and that obtained 4 h after 900 mg ractopamine. New peaks relative to the control were evident at m/z 302 (ractopamine) and 284 (instrument-induced dehydration of ractopamine), as well as 316, 382, 396, 415, 478, 492 and 528. Comparison of the 4 h range with the same spectrum without enzyme treatment (unhydrolysed) and with 0 h control revealed additional peaks at m/z 460, 497 and 500. Substantial reduction in peak height or loss of peaks on enzyme treatment thus suggested glucuronide and/or sulphate involvement for the m/z 460, 478, 492, 497, 500 and 528 species.

The pattern of ion fragments of the product ion spectrum of the m/z 478 urine metabolite in the absence of enzyme hydrolysis was consistent with that of a glucuronide formed at one of the phenolic hydroxyls, as seen in Figure 1 on the C-10′ hydroxyl (type A) or the C-10 hydroxyl (type B). Peak assignments are consistent with both such structures, and the spectrum may represent a mixture.

Similar consideration of the m/z 492 product ions suggested involvement of methylation along with glucuronidation. Ready dehydration from this peak to an m/z 474 fragment rules out methylation at the benzylic alcohol, again favouring substitution at the phenolic hydroxyls. Thus, we are given the choice of methylation at C-10/glucuronidation at C-10′ (type A), or methylation at C-10′/glucuronidation at C-10 (type B). Although the peak assignments were consistent with such structures, several (eg m/z 354 and 164) favour the Type A arrangement. A mixture of the 2 is nonetheless not ruled out.

Although there is a small trace of the m/z 316 ion in unhydrolysed urine, its peak intensity increased roughly 4-fold following hydrolysis. It also bore a +14 amu relationship, to ractopamine parent drug, making it an excellent candidate for a simple methylated species, the majority of which was released enzymatically from a mixed glucuronide/ methylated metabolite. Product ion analysis for m/z 316 indicated many peaks in common with ractopamine parent drug; such common peaks were also seen to varying extents with the glucuronides. Peak assignments for the m/z 316 metabolite are consistent with the possibility of a methylated species on either the C-10 (type A) or C-10′ (type B) phenolic alcohols.

Ractopamine metabolites by GC/MS

Ractopamine 4-h post dose metabolites were examined by GC/MS. The total ion chromatogram for derivatised SPE-extracted enzyme-hydrolysed urine indicated m/z 267 to be the major ion to focus on. This ion is derived from single internal cleavage of tris[TMS]ractopamine beyond the benzylic carbon to provide a TMS-O-C_6H_4CH-O-TMS fragment, although formally 3 fragmentations (to release TMS, and 2 O-TMS) provide other possible approaches. One discovery was that the principal peak at 9.6 min RT was a match to the standard ractopamine, released in this case by beta-glucuronidase treatment, and the basis for GC/MS confirmation as described below. The highest mw ion at m/z 502 then matches demethylated tris[TMS]ractopamine. The second discovery elicited by the ion chromatography was the occurrence of ractopamine-related metabolites as roughly equivalent area pairs of peaks. This emphasised the diastereomeric nature of the parent drug, which itself gave the appearance of 2 nearly superimposed peaks at 9.54 and 9.57 min RT.

ELISA results

A horse was given 300 mg ractopamine orally, and urine samples were collected pre- and post administration. The samples were analysed by ELISA along with a set of ractopamine standards as described in Materials and Methods.

The resultant standard curve for ractopamine in assay buffer was sigmoidal with a substantial linear portion typical of ELISA assays. The linear portion of this curve (1, 5, 10, 50, 100 ng/ml) had an I_{50} of about 10 ng/ml and an r value of 0.9811. This 5-point standard curve was used to estimate the concentration of apparent ractopamine in the urine samples (pre- and post administration) and a panel of 5 urine samples from undosed horses. All urine samples were diluted 1:4 with assay buffer prior to assay. The mean concentration of the blank urine samples (n=6) was 13.4 ng/ml ±

3.6 sem. The horse urine samples from the dosed horse were diluted and analysed by ELISA without enzyme hydrolysis.

The I_{50} of ractopamine in buffer for the TCC kit was about 10 ng/ml. Indication of a sample suspected of containing drugs is usually (ie, when the maximum OD is approximately 1.0) based on about 20% inhibition in ELISA work, which for ractopamine in buffer was achieved at about 50 ng/ml on examination of the standard curve. This value may therefore be considered the effective screening limit of detection for ELISA. As discussed below, the GC/MS limit of detection for ractopamine in urine was found to be 25–50 ng/ml. Therefore the GC/MS method reported here should be capable of confirming ractopamine positives detected by ELISA screening.

GC/MS confirmatory method for ractopamine in urine

The tris(TMS)-ractopamine derivative eluted at 9.51 min in GC/MS and the internal standard isoxsuprine-bis(TMS) eluted at 7.61 min. Of the 3 major ractopamine ions suitable for quantitative SIM, the m/z 267 ion was found to be free of interference from urinary matrix components at the retention time of ractopamine. A method was elaborated which utilised the m/z 267 ion for quantitation, ions m/z 250 and 179 for verification and m/z 178 ion of isoxsuprine-bis(TMS) as the internal standard quantifier ion. The confirmatory method was applied to an 8 h post dose urine sample. The peak of chromatographically-merged ractopamine diastereomers at 9.5 min displayed nesting of ion chromatograms at m/z 267 >250 > 179 > 502. The linearity of the standards between 10 and 500 ng/ml with its corresponding equation had an acceptable R^2 = 0.9992. Paired diastereomeric metabolite peaks generally display ions m/z 267 and 179 of the ractopamine SIM series and are distinguished as having nearly equivalent m/z 267 and 179 peak areas, or m/z 267 > 179.

The time course of ractopamine urinary excretion for a selected horse was followed. If the m/z 267 ion is assumed to reflect individual metabolite peaks with equal representation to that of ractopamine, metabolite concentrations can be estimated readily by comparison to the ractopamine standard curve. The 10.5–10.6 min component seems to mirror the ractopamine peak, supporting its assignment as a ractopamine-(TMS) derivative. Application of the resultant method enabled identification of ractopamine in enzyme-hydrolysed equine urine at a concentration of 360 ng/ml 24 h after oral administration of 300 mg, confirming the appreciable amounts seen by ELISA. The confirmation limit for ractopamine in urine is sufficient for regulatory. purposes because it matches that of the ELISA screening test.

Standard curves based on the m/z 267 ion showed a linear response for ractopamine concentrations of 10 –100 ng/ml with a correlation coefficient r>0.99, while standards in the concentration range 10–1000 ng/ml were fit to a second-order regression curve with r>0.99. Extraction efficiency for SPE ranged from 40–50%. To determine the instrument lower limit of detection (LOD), signal to noise ratios (S/N) were calculated for decreasing amounts of ractopamine-tris(TMS) until S/N for the ractopamine quantitative and qualifier ions fell below 3 at 100 pg on-column. The lower LOD for ractopamine in urine, defined as the lowest concentration at which its identity could be confirmed by comparison of diagnostic ion ratios, was 25–50 ng/ml. Accuracy and precision were determined by examining runs performed on 4 different days at concentrations of 50 and 500 ng/ml. Means and coefficients of variation, respectively, were 44.9 ng/ml ± 15.3% and 507.2 ng/ml ± 9.1%.

To assess the specificity of the GC/MS SIM method, 6 unextracted compounds were analysed at the equivalent of 250 ng/ml with and without ractopamine at the same concentration. Dobutamine, fenoterol, nylidrin and ritodrine were chosen due to their similarity in structure or known cross-reactivity in ELISA (Haasnoot *et al.* 1994; Wicker *et al.* 1995; Shelver and Smith 2000, 2002; Shelver *et al.* 2000; Smith *et al.* 2000); phenylbutazone and furosemide were chosen for their common occurrence as positives in racing chemistry. None of the TMS-derivatives co-eluted with ractopamine-tris(TMS). None of the compounds had significant effect on quantitative results for ractopamine. A TMS-derivatised contaminant of ritodrine produced trace chromatographic peaks at the retention time of ractopamine quantitative and qualifier ions. Examination of ion ratios (m/z 250, 100%; 267, 240%; 179, 255%) precluded mistaken attribution of these peaks to ractopamine (m/z 250, 100%; 267, 110%; 179, 86%), and calculated response based on m/z 267 areas at 250 ng/ml was below the minimum level of quantitation for ractopamine.

Discussion

As recognised by the ARCI, ractopamine may be able to affect racehorse performance significantly, both via its beta-adrenergic agonist properties and its anabolic activities. Therefore, it is important to assess screening methodologies such as ELISA tests, as well as the equine metabolism of this drug,

to provide target structures for mass spectrometric confirmation of its presence in urine samples. ELISA testing with a TCC ractopamine kit provided adequate linearity and sensitivity. Future ELISA experiments should perhaps be designed to assess the effects of glucuronidase treatment on detectability, given the differential sensitivity of ELISA tests for certain glucuronide stereoisomers (Shelver and Smith (2000). GC/MS SIM confirmation was also adequate for quantitation of ractopamine following glucuronidase release of conjugates up to 24 h post dose, and matched the profile of urine excretion provided by ELISA.

Equine ractopamine metabolism consisted predominantly of the formation of phase II metabolites in keeping with other species, including turkey (Smith *et al.* 2000) where the C-10 glucuronide is divided roughly equally between bile and urine; rat (Smith *et al.* 1995) where a C-10′-sulfate/C-10-glucuronide bis-conjugate was identified in bile; and cattle, sheep and ducks (Smith 1998; Smith and Shelver 2002), wherein urine hydrolysable conjugates have been indirectly described. Metabolism of ractopamine is diverse with glucuronidation, sulphation and methylation routes available to it. We speculate that, despite this observed diversity, there is no particular bias of metabolism type towards any stereochemical configuration, owing to the equivalent peak areas revealed by GC/MS analysis of metabolites arising from the pharmaceutical RR, SS, RS, SR mixed diastereomeric composition.

Acknowledgements

Published as Kentucky Agricultural Experiment Station Article 04-14-008 with the approval of the Dean and Director, College of Agriculture and Kentucky Agriculture Experiment Station.

Publication #337 from the Equine Pharmacology, Therapeutics and Toxicology Program of the Maxwell H. Gluck Equine Research Center, University of Kentucky, Lexington, KY 40546-0099.

Supported by a grant from The Kentucky Racing Commission and the Kentucky Equine Drug Research Council, Lexington, KY.

This analytical method has been certified for transferability by the Pennsylvania Equine Toxicology and Research Laboratory, West Chester, PA, an A2LA-accredited laboratory.

References

Anderson, P.T., Helferich, W.G., Parkhill, L.C., Merkel, R.A. and Bergen, W.G. (1990) Ractopamine increases total and myofibrillar protein synthesis in cultured rat myotubes. *J. Nutr.* **120,** 677-683.

Antignac, J.P., Marchand, P., LeBizec, B. and Andre, F. (2002) Identification of ractopamine residues in tissue and urine samples at ultra-trace level using liquid chromatography-positive electrospray tandem mass spectrometry. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **774,** 59-66.

Bosken, J.M., Lehner, A.F., Hughes, C.G., Woods, W.E., Camargo, F.C., Harkins, J.D., Boyles, J. and Tobin, T. (2004) A GC/MS method for quantitation of isoxsuprine in biological fluids of the horse utilising electron impact ionization. *J. anal. Toxicol.* **28,** 27-34.

Churchwell, M.I., Holder, C.L., Little, D., Preece, S., Smith, D.J. and Doerge, D.R. (2002) Liquid chromatography/electrospray tandem mass spectrometric analysis of incurred ractopamine residues in livestock tissues. *Rapid Comm. Mass Spectrom.* **16,** 1261-1265.

Haasnoot, W., Stouten, P., Lommen, A., Cazemier, G., Hooijerink, D. and Schilt, R. (1994) Determination of fenoterol and ractopamine in urine by enzyme immunoassay. *Analyst* **119,** 2675-2680.

Helferich, W.G., Jump, D.B., Anderson, D.B., Skaerlund, D.M., Merkel, R.A. and Bergen, W.G. (1990) Skeletal muscle alpha-actin synthesis is increased pretranslationally in pigs fed the phenethanolamine ractopamine. *Endocrinology* **126,** 3096-3100.

Ricke, E.A., Smith, D.J., Feil, V.J., Larsen, G.L. and Caton, J.S. (1999) Effects of ractopamine HCl stereoisomers on growth, nitrogen retention, and carcass composition in rats. *J. Anim. Sci.* **77,** 701-707.

Shelver, W.L. and Smith, D.J. (2000) Development of an immunoassay for the beta-adrenergic agonist ractopamine. *J. Immunoassay* **21,** 1-23.

Shelver, W.L., Smith, D.J. and Berry, E.S. (2000) Production and characterization of a monoclonal antibody against the beta-adrenergic agonist ractopamine. *J. agric. Fd. Chem.* **48,** 4020-4026.

Shelver, W.L. and Smith, D.J. (2002) Application of a monoclonal antibody-based enzyme-linked immunosorbent assay for the determination of ractopamine in incurred samples from food animals. *J. agric. Fd Chem.* **50,** 2742-2747.

Smith, D.J., Giddings, J.M., Feil, V.J. and Paulson, G.D. (1995) Identification of ractopamine hydrochloride metabolites excreted in rat bile. *Xenobiotica* **25,** 511-520.

Smith, D.L. (1998) The pharmacokinetics, metabolism, and tissue residues of beta-adrenergic agonists in livestock. *J. Anim. Sci.* **76,** 173-194.

Smith, D.J., Feil, V.J. and Paulson, G.D. (2000) Identification of turkey biliary metabolites of ractopamine hydrochloride and the metabolism and distribution of synthetic [14C]ractopamine glucuronides in the turkey. *Xenobiotica* **30,** 427-440.

Smith, D.J. and Shelver, W.L. (2002) Tissue residues of ractopamine and urinary excretion of ractopamine and metabolites in animals treated for 7 days with dietary ractopamine. *J. Anim. Sci.* **80,** 1240-1249.

Turberg, M.P., Rodewald, J.M. and Coleman, M.R. (1996) Determination of ractopamine in monkey plasma and swine serum by high-performance liquid chromatography with electrochemical detection. *J. Chromatogr.* **675,** 279-285.

Wicker, A.L., Turberg, M.P. and Coleman, M.R. (1995) Evaluation of ractopamine cross-reactivity in several commercially available beta-agonist enzyme immunoassay kits. *Analyst* **120,** 2879-2881.