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CONFIDENTIAL

Title: **Metabolism of the beta-agonist ractopamine in the horse.**

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Abstract

We have investigated the detection, confirmation and metabolism of the beta-adrenergic agonist drug ractopamine in the horse. A Testing Components Corporation ELISA kit for ractopamine provided a linear portion of the dose response curve with an I-50 of 10 ng/ml and a limit of detection of 50 ng/ml. The kit was readily able to follow ractopamine equivalents in unhydrolyzed urine up to 24 hours following a 300 mg oral dose. GC/MS confirmation comprised glucuronidase treatment, solid phase extraction and trimethylsilyl derivatization, with selected ion monitoring of ractopamine-tris(TMS) m/z 267, 250, 179 and 502 ions. Quantitation was elaborated in comparison to a ritodrine-tris(TMS) internal standard monitored similarly. Sensitivity of this analytical method was adequate, with a limit of detection of approximately 30 ng/ml depending on the presence of an interfering component. Urine concentration 24 hours post-dose was measured at 360 ng/ml after oral administration of 300 mg. Urinary metabolites were identified by electrospray ionization (+) tandem quadrupole mass spectrometry, and were shown to include glucuronide, methyl, and mixed methyl-glucuronide conjugates, as well as an unusual conjugate adding 113 amu to give an m/z 415 [M+H] species or two times 113 amu to give an m/z 528 [M+H] species with a related daughter ion mass spectrum. These results suggest that glucuronide hydrolysis followed by parent drug recovery and TMS derivatization is the optimal pathway for detection and confirmation of ractopamine in equine urine.

Introduction

Ractopamine *N*-[2-(4-hydroxyphenyl)-2-hydroxyethyl]-1-methyl-3-(4-hydroxyphenyl)propylamine is a beta-adrenergic agonist marketed under the trade name Paylean® (1) which is approved for use in swine and other livestock as a growth regulator. It has a phenethanolamine structure and has repartitioning properties enabling reduction of fat, increased muscle mass, and improved feed utilization efficiency in swine, cattle, and turkeys (2 and refs therein). Ractopamine promotes protein deposition with little effect on fat disposition in the pig (3,4). Dietary ractopamine decreases basal plasma insulin concentrations but has no effect on plasma glucose or non-esterified fatty acids (4). Anti-lipolytic effects of insulin tend to be augmented as well (4). Dietary ractopamine has also been shown to decrease lipolytic responses to fenoterol, evident after four dose treatments, and the hyperinsulinaemic response to fenoterol was attenuated by dietary ractopamine (4). The de-sensitization of adipose tissue beta-adrenergic receptors is consistent with the observations that dietary ractopamine has little effect on the rate of fat deposition in the growing pig (4).

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 (5). In rats, ractopamine increased methionine incorporation in cultured muscle cells (6). Studies in rats indicate that of the four possible ractopamine stereoisomers (RR, RS, SR, SS), the RR isomer is responsible for the majority of leanness-enhancing effects (7).

Both conjugated and nonconjugated metabolites of ractopamine have been identified in various species. In cattle and sheep, for example, ractopamine residues in urine samples were measured before and after hydrolysis of conjugates. On withdrawal day 0 following extended administration, concentrations of parent ractopamine in sheep urine were ~9.8 ppb and were below the LOQ (5 ppb) beyond the 2-day withdrawal period (8). After hydrolysis of conjugates, ractopamine concentrations were ~5300 ppb on withdrawal day 0 and ~180 ppb on withdrawal day 7 (8). A similar picture emerges on study of ractopamine concentrations in cattle urine. The data indicate that after the hydrolysis of conjugates, ractopamine should be detectable in urine of sheep as long as 7 days after the last exposure to ractopamine and as long as 5 days after withdrawal in cattle (8).

Conjugate metabolite structures have been studied. In rat bile, for example, approximately 46% of biliary metabolites were identified as a sulfate-ester, glucuronic acid diconjugate of ractopamine. The site of sulfation was at the C-10' phenol (phenol attached to carbinol) and glucuronidation was at the C-10 phenol (phenol attached to methylpropylamine) of ractopamine (9). Monoglucuronides conjugated to the phenols at C-10 (60%) or C-10' (13%) have also been found in turkey poults (10,11). Regioselective glucuronidation occurred favoring the C-10 phenol of ractopamine, with specificity for (1R,3R) and (1R,3S) stereoisomers (11).

Enzyme immunoassay-based detection of ractopamine has been made possible through the availability of commercial ELISA kits (12-15), but in at least one case (RS, SR) glucuronides showed only 4% cross-reactivity, whereas (RR, SS) diastereoisomer glucuronides conjugated at the same phenolic group showed no detectable reactivity with the antibody (13). Shelver and Smith have nonetheless been able to demonstrate applicability of the monoclonal antibody-based ELISA for determination of ractopamine residues in sheep and cattle samples (16).

Ractopamine is an Association of Racing Commissioners, International (ARCI) Class 3 drug (17), and is therefore a recognized therapeutic agent, although of unlikely therapeutic value in the racing horse, yet with a potential for abuse owing to a certain likelihood to affect performance. Therefore, illegal use of ractopamine needs to be monitored, and its discovery in performance horses may lead to sanctions against owners or trainers.

The purpose of this study is to determine the metabolism of ractopamine in the horse with the intention of providing the structure(s) of target metabolites for mass spectrometric confirmation of ractopamine dosing. HPLC methods with various detectors exist for ractopamine (8, 18), however electrospray ionization mass spectrometry is rapidly becoming more widespread and with proliferation of methodologies (19,20). In addition to mass spectrometric analyses, we determined detectability on a commercial ELISA kit.

Materials and Methods

Three mature Thoroughbred mares, weighing 564, 542, and 550 kg were acclimated to their stalls 24 h prior to experimentation. The animals 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. The animals were vaccinated annually for tetanus and were dewormed quarterly with ivermectin (MSD Agvet, Rahway, New Jersey, USA). A routine clinical examination was performed before each experiment to assure that the animals were healthy and sound. During experimentation, horses were provided water and hay ad libitum. Each mare served as its own control. Animals used in these experiments were managed according to the rules and regulations of the Institutional Animal Care Use Committee at the University of Kentucky, which also approved the experimental protocol Ractopamine (900 mg) was administered to horses, orally, in the form of the feed supplement Paylean® (Elanco, Charlotte, NC). Urine samples were collected immediately before and at 4 h after administration using a Harris flush tube (24 Fr x 60 in; Seamless, Ocala, Florida, USA). Urine samples were divided into aliquots stored at -20°C until assayed.

Sample collection and preparation

The ractopamine standard for mass spectral analysis was purchased as ractopamine-HCl (USDA). Pre- and post-administration urine samples were treated by solid phase extraction. 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 (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, Beverly, Massachusetts, USA).

ELISA Detection

Testing Components Corp. (TCC, Collinsville, IL) ractopamine ELISA kits were used to establish the detection limit for ractopamine in equine urine. A stock solution (1mg/ml) of ractopamine was prepared in methanol. ELISA standards (0.1 to 1000 ng/ml) were prepared by dilution of stock with ELISA kit assay buffer. The ELISA kit consisted of microtiter 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 (and/or samples) into the appropriate wells. Antibody #1 solution (100µl) was added to each well followed by gentle mixing and incubation for 30min at 37°C. The plate was then washed 3 times with diluted wash solution (300µl). Freshly diluted antibody #2 (150µl) was added to each well with gentle mixing. The plate was incubated 30min at 37°C. The plate was again washed 3 times as above. Substrate (100µl) was added to each well, and the OD at 650nm was read with an ELX800 Microplate Reader (BioTek Instruments, Inc.) at 15min.

Beta-glucuronidase hydrolysis

For beta-glucuronidase hydrolysis, the urine samples were treated for 3 h at 65°C with *Patella vulgata* beta-glucuronidase (1000 units of Sigma Type L-II/mL urine brought to 0.25 M sodium acetate, pH 5). The resultant hydrolysates were subjected to solid phase extraction as described below.

Solid Phase Extraction and Derivatization

Solid phase extractions were run on a Zymark RapidTrace® automated system. SPE columns were conditioned by adding sequentially 3 ml methanol, 3 ml water, 1 ml 0.1M sodium phosphate buffer, pH 6.0. Samples were loaded, then the column washed sequentially with 2ml water, 2ml 1M acetic acid, 4 ml methanol. The column was dried with N_2 for 1 minute. Then, the column was eluted with 3ml dichloromethane/isopropanol/NH_4OH (concentrated) (78:20:2) 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 in 15 μL of N,N-dimethylformamide and 50 μL of BSTFA-1% TMCS (Pierce Chemicals, Rockville, IL) and immediately transferred to a micro-injection vial. Derivatization occurred during incubation at 70°C for 30 min. One μL was injected into the GC/MS. In cases where ESI (+) MS analysis was the preferred method, resuspension 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 derivatization was accomplished by GC/MS selected ion monitoring (SIM). The GC column was a HP-5 MS, 30 m x 0.25 mm x 0.25 μm film thickness operated in the splitless mode with 1 ml/min helium. The GC oven temperature was programmed as follows: 180°C for 2 min, then increased to 280°C at 20°C/min, held at 280°C for 10 minutes. Data were collected following a 1 μl injection as follows. Ritodrine was chosen as an internal standard due to its successful use in isoxsuprine analysis (J. Bosken, unpublished results). The m/z 236 ion of tris(TMS)-ritodrine was monitored as an internal standard. The major ions of tris(TMS)-ritodrine are m/z 236, 73, 193, and 267 in order of decreasing abundance, with a small m/z 488 ion resulting from loss of CH_3 from the molecular ion. Ions monitored for the tris(TMS)-ractopamine derivative were m/z 267, 250, 179, and 502, again in order of decreasing abundance. The origin of these major ions may be explained by scission of the molecular ion to release TMS-O-C_6H_4-CH-O-TMS (m/z 267), $CH_2NHCH(CH_3)CH_2CH_2C_6H_4$-O-TMS (m/z 250) or $CH_2C_6H_4O$-TMS (m/z 179) fragments. Preparation of the ractopamine standard curve was accomplished by determining the internal standard (m/z 236) and ractopamine (m/z 250) peak areas for a series of standards, calculating the ratio of internal standard area/ractopamine area on the horizontal axis, and plotting concentrations as a function of

this ratio. Standard curves prepared in this fashion provided correlation coefficient R^2>0.99. For GC/MS scanning experiments, the m/z 50-700 mass range was scanned at 1.19 scans/sec.

MS/MS analysis

Full scan electrospray ionization (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 optimized 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). The peak shape and intensity of the monoprotonated ractopamine m/z 302 ion were optimized 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 optimizing settings as needed for the second quadrupole. Generally, the collision gas was set to 1-3 X 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 24 V, the collision energy was set at –20 V, the capillary of the ESI probe was set at +3.0 k V, the skimmer at 2.1 V, and the HV lens was set at 0.54 kV. Source temperature was set at 120°C.

Results

Ractopamine by Electrospray(+) MS

Ractopamine, 301 m.w., readily produced a M+H pseudomolecular ion at m/z 302 when dissolved in acetonitrile:0.05% formic acid and examined by ESI(+)MS, as shown in Fig. 1 [top]. The daughter ion spectrum of this m/z 302 species gave intense responses particularly at m/z 107, 121, 136 and 164 [Fig. 1, bottom], and these peaks could be readily assigned in keeping with the structure of ractopamine (see assignments in the figure legend). Urine collected on the second day of a repeated dosing regimen four hours post-dose was subjected to beta-glucuronidase treatment. The daughter ion spectrum of the solid phase-extracted (SPE) m/z 302 material is shown in Fig. 2 and is a match to that in Fig. 1, bottom. The Fig. 1 daughter ion spectrum is also a perfect match for that seen from a pronounced m/z 302 peak in the commercial formulation Paylean® (data not shown), which is important in verifying that no prodrugs or unintended conjugates were present in the feed version of the drug.

Ractopamine metabolites by ESI(+)MS

ESI(+) mass spectrometry revealed additional metabolites of ractopamine in urine by comparison of glucuronidase-treated SPE extractions at 0 hour and 4 hours following the initial dose of ractopamine. Fig. 3 displays such a comparison, and new peaks are evident at m/z 302 (ractopamine) and 284 (instrument-induced dehydration of ractopamine, as seen also in Fig. 1, top), as well as 316, 478, 492 and 528. Fig. 4 shows an expansion of the m/z 410-570 region of the spectrum emphasizing these latter species; comparison of the 4 hour range with the same spectrum without enzyme treatment (unhydrolyzed) reveals additional peaks at m/z 460, 497 and 500. Drastic reduction in peak height or elimination on enzyme treatment thus suggests glucuronide and/or sulfate involvement for the m/z 460, 478, 492, 497, 500 and 528 species.

Examination of the daughter ion spectrum of the m/z 478 urine metabolite in the absence of enzyme hydrolysis is presented in Fig. 5. The pattern of ion fragments is consistent with that of a glucuronide formed at one of the phenolic hydroxyls, as depicted in the figure on the C-10' hydroxyl (type A) or the C-10 hydroxyl (type B). Peak assignments are consistent with such structures, and the spectrum could well represent a mixture, although several of the minor peaks seem to favor the type A structure. The m/z 460 ion seen on direct scan (Fig. 4, top) thus represents a dehydrate originating from the unconjugated benzylic alcohol of the m/z 478 glucuronide(s).

Similar examination of the m/z 492 daughter ions is presented in Fig. 6. Its 14 amu difference from the 478 peak, and the number of common peaks between Figs. 5 and 6 suggest involvement of methylation along with glucuronidation. Ready dehydration from this peak to an m/z 474 fragment tends to rule out methylation at the benzylic alcohol, again favoring 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). While the peak assignments given in the figure legend are consistent with such structures, many of them again favor the type A arrangement. A mixture of the two is nonetheless not ruled out.

While there is a small trace of the m/z 316 ion present in unhydrolyzed urine, its peak intensity increases roughly four-fold following hydrolysis. Since it is also bears a +14 amu relationship--this time to ractopamine parent drug--it is an excellent candidate for a simple methylated species released enzymatically from a mixed glucuronide/methylated metabolite. Daughter ion analysis for m/z 316 is presented in Fig. 7 and indicates many peaks in common with ractopamine parent drug, seen on comparison with Fig. 1; such common peaks were also seen to varying extents with the glucuronides (Figs. 5 and 6). 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. However, there is more ambiguity as to whether assignments favor one type over another in this case.

The m/z 528 peak is curious in that it may very well be related to an additional peak that was at first thought to be a matrix ion that increased dramatically in the four-hour urine sample, specifically m/z 415 (Fig. 3). Fig. 8 shows that both the m/z 528 and 415 share significant ractopamine-related ions, m/z 302, 284, 164. In addition, the m/z 528 spectrum includes m/z 415 as a daughter ion. If the peaks are related as asserted, then m/z 415 adds a net 113 amu to [M+H] ractopamine at m/z 302, and 528 must represent the execution of two such additions. Consideration of possible structure(s) for this conjugation reaction will be presented in the Discussion section.

Figure 9 presents a parent ion scan for the ractopamine m/z 164 ion that comprises a $NHCH(CH_3)CH_2CH_2C_6H_4OH$ fragment. Without enzymatic hydrolysis (Fig. 9, top), the spectrum shows conjugates m/z 478 and 492 [trace], the 478 dehydrate at m/z 460, and small amounts of parent drug at m/z 302 and its 284 dehydrate. Beta-glucuronidase treatment [bottom] reveals these same peaks in dramatically changed proportions, and confirms the relationship to ractopamine of ions m/z 284, 302, 460, 478 and 492, as well as the newly-revealed peaks m/z 316, 415 and 528.

Ractopamine metabolites by GC/MS

Figure 10 presents an examination of ractopamine 4-hour post-dose metabolites by GC/MS. The top figure shows the total ion chromatogram for derivatized SPE-extracted enzyme-hydrolyzed urine, whereas the middle panel shows a much more simplified pattern derived by performing ion chromatographic analysis of the data in the top panel focusing on ion m/z 267. This ion is derived from internal cleavage of tris[TMS]ractopamine to provide a TMS-O-C_6H_4CH-O-TMS fragment. Table 1 lists predicted molecular weights for ractopamine metabolites and their TMS derivatives, and this provided a guide to interpretation of this data. One discovery is that the principal peak at 9.6 min RT is a match to the standard ractopamine, released in this case by beta-glucuronidase treatment. The highest m.w. ion at m/z 502 then matches the demethylated tris[TMS]ractopamine listed in Table 1. The second discovery elicited by the Fig.10 ion chromatography is the occurrence of ractopamine-related metabolites as roughly equivalent area pairs of peaks. This emphasizes the diastereomeric nature of the parent drug, which itself gives the appearance of two nearly superimposed peaks at 9.54 and 9.57 min RT. Table 2 lists corresponding pairs of peaks based on highly similar mass spectra and their likely structural assignments based on consideration of ESI(+)MS data and predictions of Table 1.

ELISA Results

A horse was dosed with 300 mg ractopamine orally, and urine samples were collected pre- and post-administration. These urine samples were analyzed 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 ractopamine in the dosed horse urine samples (pre- and post-administration) and a panel of 5 undosed horse urine samples. All urine samples were diluted 1:4 with assay buffer prior to assay. The mean concentration of the blank urine samples (n=6) was 13.4ng/ml ±3.6 SEM. The dosed horse urine samples were diluted and analyzed by ELISA without enzyme hydrolysis. An estimate of the apparent ractopamine concentrations in these samples is shown in Figure 11 (left). The sigmoidal standard curve for ractopamine over a five-log scale is shown in Fig. 11 (right).

The I-50 of ractopamine in buffer for the TCC kit was about 10ng/ml. Indication of a sample as suspected drug-containing is usually (i.e., when the maximum O.D. 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 ELISA limit of detection. As discussed below, the GC/MS limit of detection for ractopamine was found to be about 10ng/ml. Therefore the GC/MS method reported here should easily confirm any ELISA ractopamine positives detected.

GC/MS Confirmatory method for ractopamine in urine

As described in Fig. 10, in GC/MS the tris(TMS)-ractopamine derivative elutes at 9.51 minutes and the internal standard trisTMS-(ritodrine) (not shown) elutes at 8.67 minutes. This method was used to identify ractopamine in enzyme-hydrolyzed equine urine at a concentration of 360 ng/ml 24 hours after oral administration of 300 mg of the drug. Endogenous compounds co-extracted from urine which appeared as a tailing shoulder on the ractopamine GC peak at high ractopamine concentrations produced significant interferences only when the drug was present in low concentrations. Of the three major ractopamine ions suitable for SIM, only m/z 250 was free of interference from urinary matrix components at the retention time of ractopamine. Though ractopamine may be detected at low concentrations using the 250 m/z ion, the urinary interference limits the capability to confirm its identity to concentrations above approximately 10-30 ng/ml in samples analyzed to date. This confirmation limit varies with the animal whose urine is being analyzed. Though the confirmation limit for ractopamine in urine was not very low, it is adequate for our purposes since the ELISA screening test for ractopamine has a detection limit of approximately 50 ng/ml. Other laboratories attempting to adapt our methods may have significantly higher or lower limits of confirmation. Attempts to improve the resolution of ractopamine from the interfering peak by altering the GC temperature program were unsuccessful.

Discussion

As a beta-agonist, ractopamine may have the ability to significantly affect race horse performance, both via its beta-adrenergic agonist properties and its anabolic activities. Therefore, it is of importance to assess both screening methodologies such as

ELISA, 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 kit provided adequate linearity and sensitivity, and Fig. 11 demonstrates the ability of this test to follow the time course of excretion following a 300 mg dose. This dose at less than 1 mg/kg is minimal, when compared to that given to pigs at around 20 mg/kg (4). Future experiments should perhaps be designed to assess the effects of glucuronidase treatment on detectability, given the differential detectability of certain glucuronide stereoisomers on ELISA tests (13). GC/MS SIM confirmation was also adequate for semi-quantitation of ractopamine following glucuronidase release of conjugates up to 24 hours post-dose.

Equine ractopamine metabolism consists predominantly of the formation of phase II metabolites in keeping with other species, including turkey (10) where the C-10 glucuronide is divided roughly equally between bile and urine; rat (9) where a C-10'-sulfate/C-10-glucuronide bis-conjugate was identified in bile; and cattle, sheep and ducks (2,8), wherein urine hydrolyzable conjugates have been indirectly described. The general scheme of ractopamine metabolism in the horse is summarized in Fig. 12, applying the simplest interpretations of the data currently available. The figure only includes the non-stereochemical image of ractopamine parent structure, as the absolute placement of glucuronides and methyl groups admittedly cannot be finalized by the information currently available. The scheme also demonstrates how the diverse metabolites signified by M+H values m/z 316, 415, (429), 478, 492 and 528 can be related to the parent m/z 302 M+H, while also accounting for the presumably instrument-derived dehydration products at m/z 284 and 460. Other ESI(+) peaks that initially seemed related but for which no support could be obtained included m/z 382, 396, 497 and 500 (data not shown).

One interesting aspect of the equine metabolism of ractopamine involves the addition of 113 amu in one or two steps via an unknown mechanism, presumably involving the two phenolic hydroxyl groups. The involvement of the phenols is supported by similar observations made recently with the structurally-related drug isoxsuprine which has a single phenolic group and adds only one 113 amu group (Lehner and Bosken, unpublished observations). Review of known generic mechanisms for Phase II conjugation reactions to various functional groups (21) currently include methylation (+14 amu), acetylation (+42 amu), sulfation (+80 amu), succinylation (+101 amu), mercapturic acid formation (+162 amu), and glucuronidation (+176 amu). Amino acid conjugation occurs in various species, generally with amide formation at a drug aromatic carboxylic acid; glycine, m.w. 75, for example, adds a net 57 amu. If unique amino acid conjugation were at play here, we suggest conjugation with the isomeric amino acids leucine and isoleucine (m.w. 131) as possibilities, primarily on the basis that such conjugation via esterification would enable a net gain of 113 amu. This would be both an unprecedented conjugation for these amino acids, and a unique form of metabolism for the horse. It offers the difficulty that such conjugates should be subjected to the effects of blood-borne esterases, unless they are unusually stable or in fact formed directly in the kidneys prior to excretion. The rate of deconjugation of bile acid amidates by

cholylglycine hydrolase is dependent on the structure of the amino acid side chain, for example (22). We speculate that, whatever metabolic intermediate is conjugated with ractopamine in the horse, it may represent an alternative route of metabolism for certain drugs, and become of greater importance during times of metabolic stress, for example during starvation, which has been found to alter relative rates of glucuronidation and sulfation in animal models (23-24).

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Figures

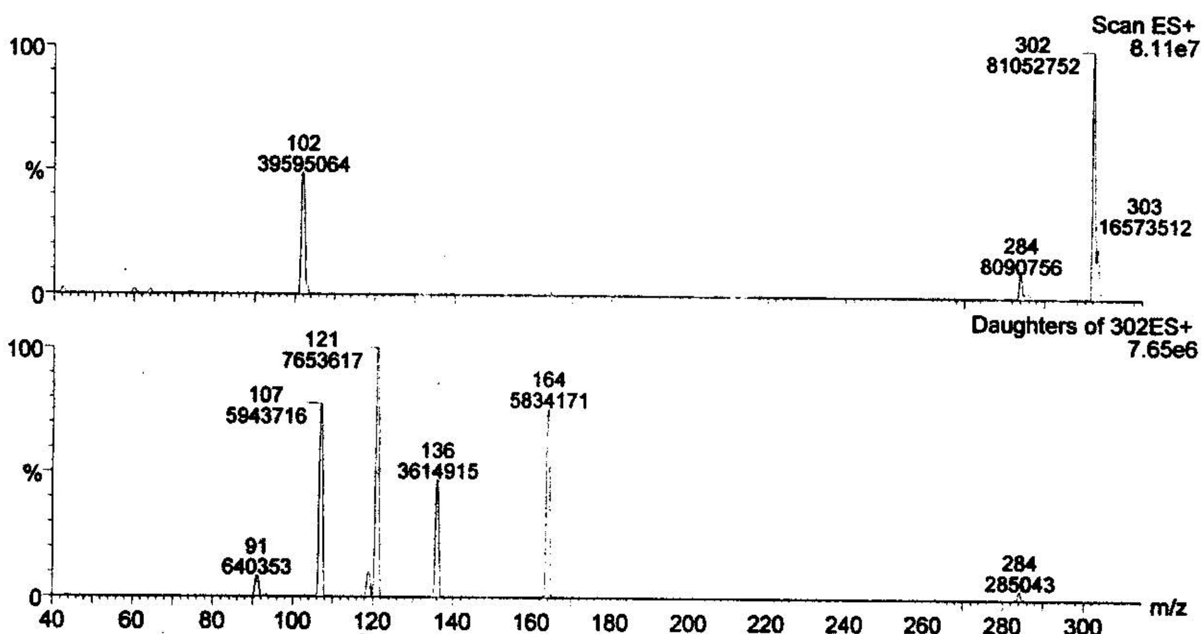


Fig. 1. Ractopamine standard: full scan [top] indicating ractopamine $[M+H]^+$ at m/z 302 and its dehydrate at m/z 284, and daughter ion spectrum by ESI(+) [bottom], 10ug/ml in acetonitrile:0.05% formic acid, 1:1, examined by direct infusion at 1.2 ml/hr.

Assignments: 284=loss of H_2O (benzylic alcohol); 164=[284 - $CH_2CH_2C_6H_4OH$, either end]+H; 136=[284 - $HOC_6H_4CH_2CH_2CHCH_3$] +H; 121=$HOC_6H_4CH_2CH_2$; 119= 284 - $HOC_6H_4CH_2CH_2CH(NH_2)CH_3$; 107=$HOC_6H_4CH_2$; 91=$HOC_6H_4CH_2CH_2$.
Minor peaks: 178 =284 - $CH_2C_6H_4OH$; 149=$HOC_6H_4CH_2CH_2CHCH_3$; 147=284 - [CH_3 and $HOC_6H_4CH_2CH_3$]; 103 = loss of [$HOC_6H_4CH_2$ and HOC_6H_4] +H; 74= $CH_3CH(NH_2CH_2)CH_3$; 58=$CH_2CH(NH_2)CH_3$; and 44 = $CH(NH_2)CH_3$; unassigned=m/z 66, 77, 79, 109.

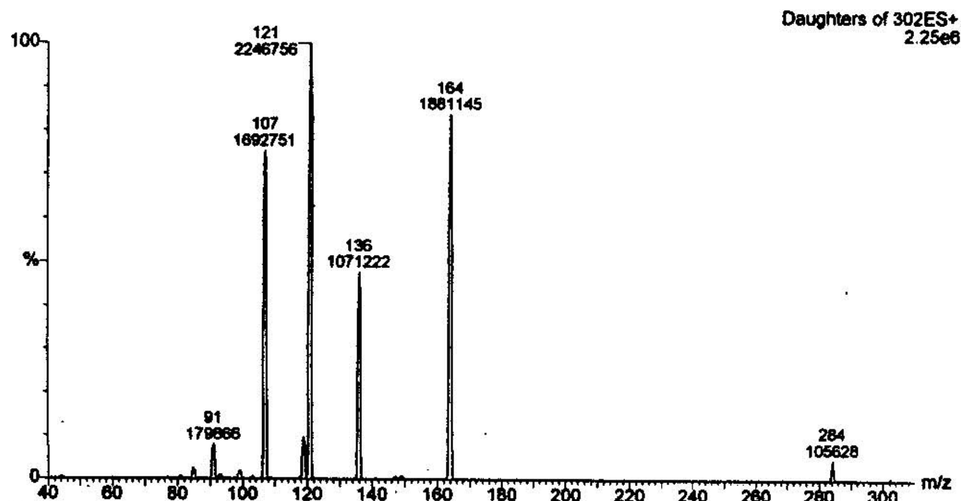


Fig. 2. Compound released from day 2, 4 hour equine urine sample by glucuronidase treatment following solid phase extraction, and matching ractopamine standard, Fig. 1.

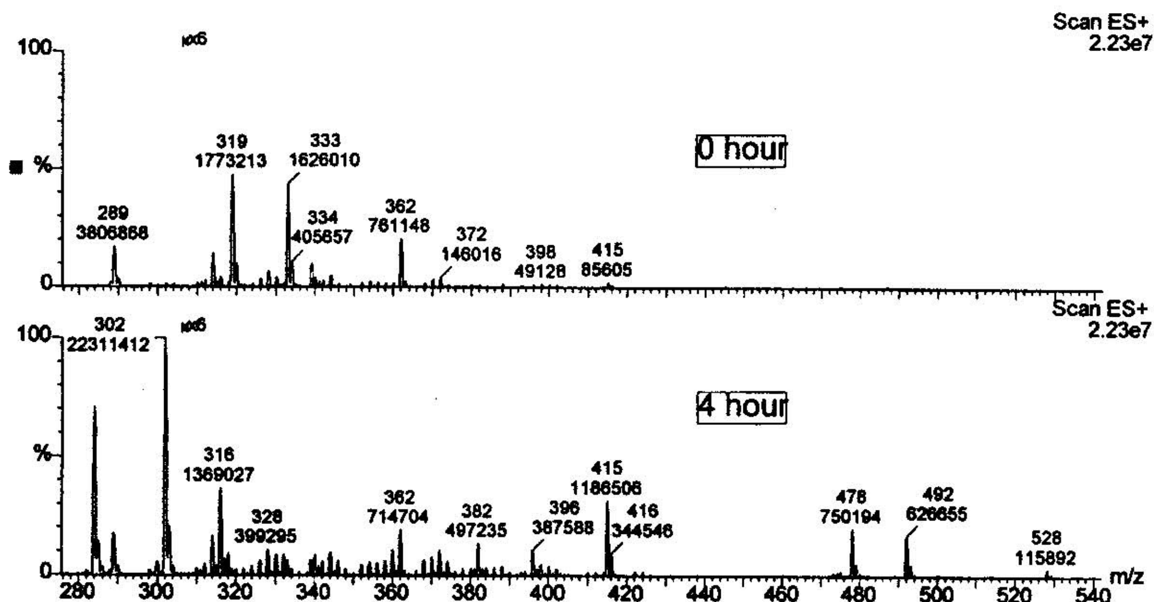


Fig. 3. Comparison of full scan ESI(+) spectra of SPE-extracted urine following glucuronidase hydrolysis, 0 hr [top] and 4 hr [bottom] after 300 mg ractopamine dose. Note new peaks at m/z 284, 302, 316 and those above 460 in the bottom spectrum.

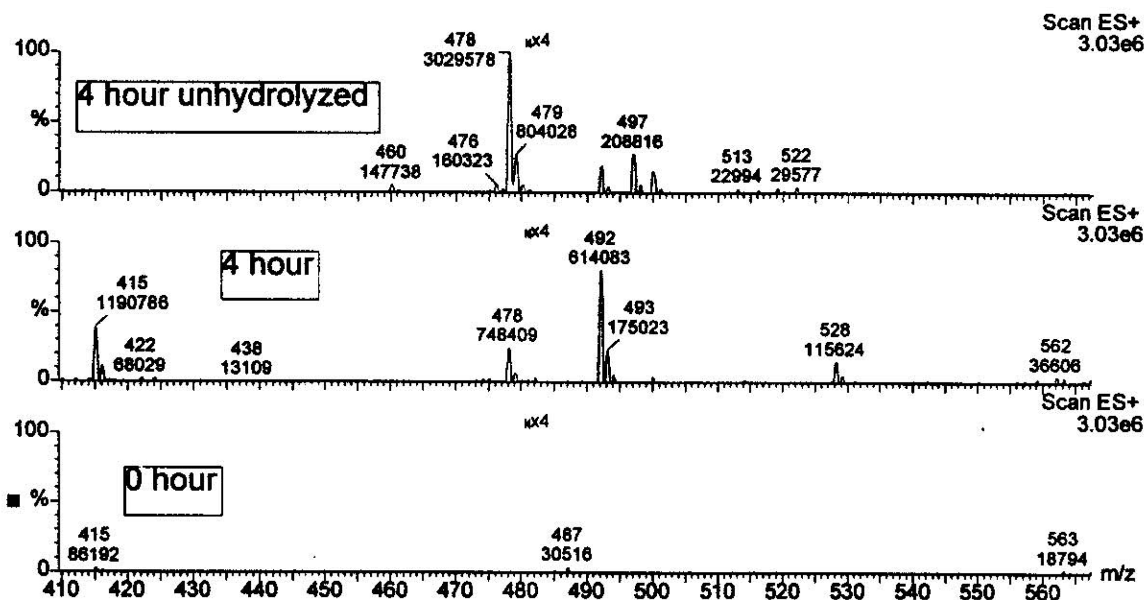


Fig. 4. Expansion of high molecular weight region for study involving 300 mg ractopamine dose. Unhydrolyzed urine 4 hour sample full scan [top] is compared with hydrolyzed result [middle] and 0 hour scan [bottom]. Molecular weight species for which pattern changes suggest glucuronide and/or sulfate involvement include m/z 460, 478, 492, 497, 500, and 528.

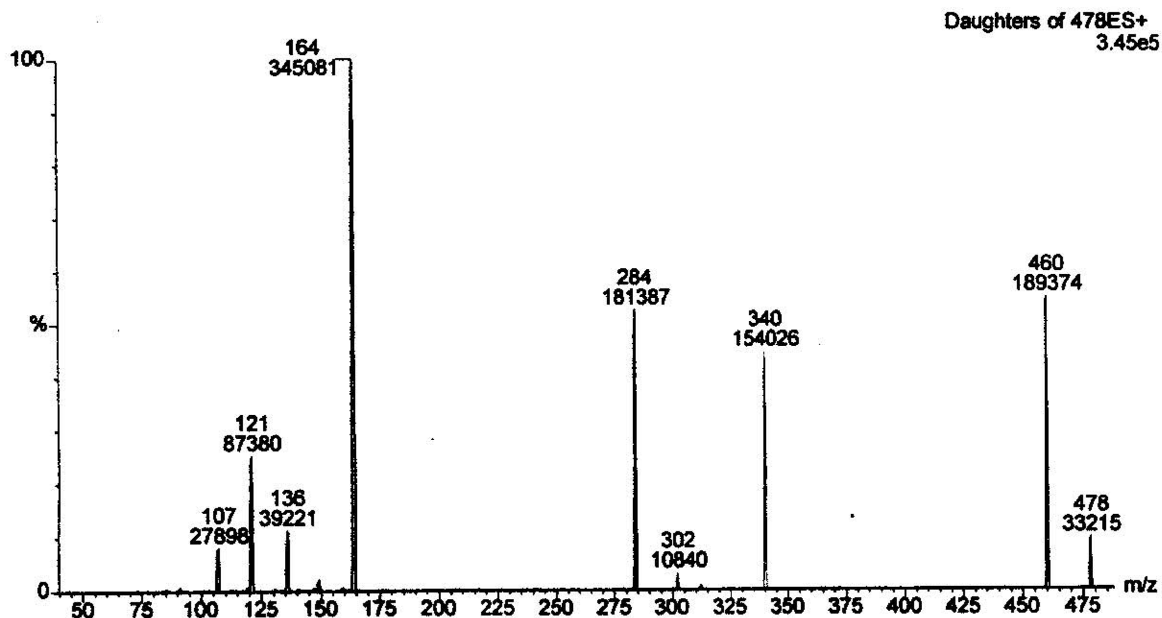
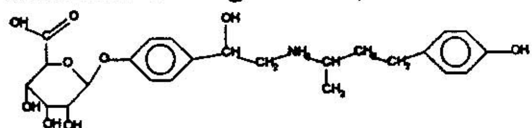
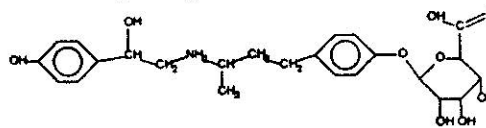


Fig. 5. Daughter ion spectrum by ESI(+) of m/z 478 ion seen in Fig. 4 [top]. The pattern is consistent with a glucuronide structure at a phenolic hydroxyl of one of the following types:





Type A Type B

Assignments: m/z 460, loss of H_2O (benzylic alcohol); 340 = [460 - $CH_2CH_2C_6H_4OH$]+H (type A) or [478 minus COOH and minus C_6H_4OH] (type B); 302 = loss of glucuronic acid moiety +H; 284 = 302 minus H_2O (benzylic alcohol); 164=[284 - $CH_2CH_2C_6H_4OH$]; 136=[284 - $HOC_6H_4CH_2CH_2CHCH_3$] +H; 121=$HOC_6H_4CH_2CH_2$; 107=$HOC_6H_4CH_2$. *Minor peaks:* 312 = [460 - $HOC_6H_4CH_2CH_2CHCH_3$] +H (type A); 159 = glucuronic acid moiety minus OH; 149=$HOC_6H_4CH_2CH_2CHCH_3$ (type A); 147 = 284 - [CH_3 and $HOC_6H_4CH_2CH_3$]; 131 = glucuronic acid moiety minus COOH; 119 = 284 - $HOC_6H_4CH_2CH_2CH(NH_2)CH_3$; 91 = $HOC_6H_4CH_2CH_2$.

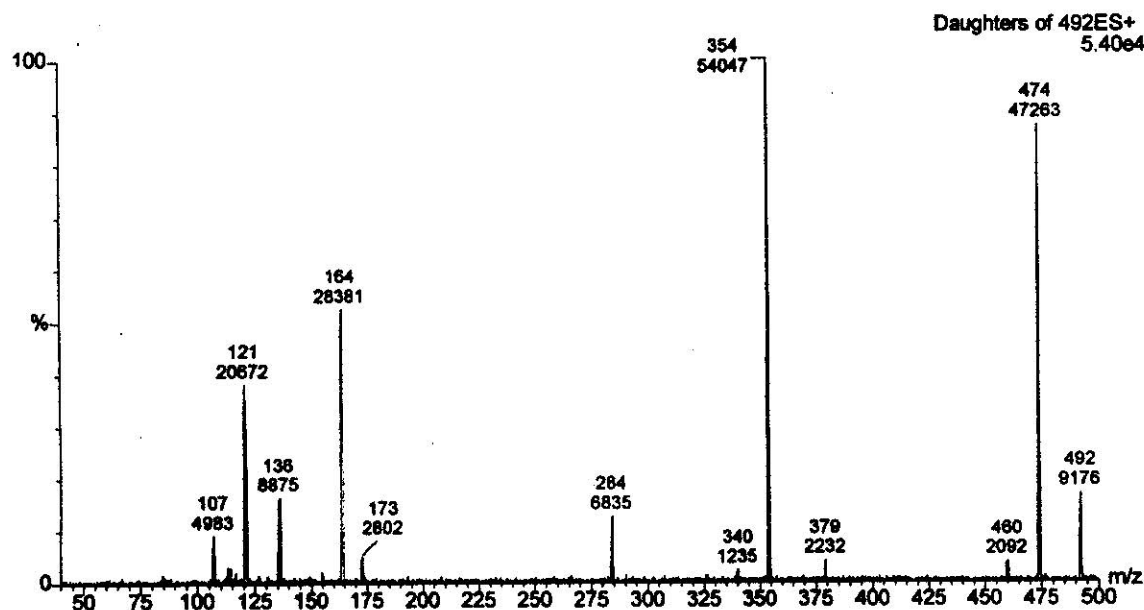
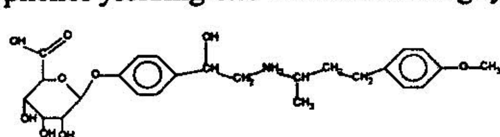
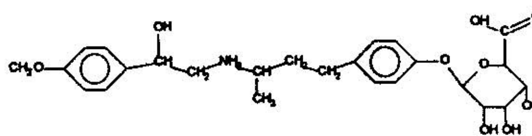


Fig. 6. Daughter ion spectrum by ESI(+) of m/z 492 ion seen in Fig. 4 [top]. The pattern is consistent with a glucuronide structure at a phenolic hydroxyl and a methylation at the other phenol yielding one of the following types:



Type A



Type B

Assignments: m/z 474, loss of H_2O (benzylic alcohol); 460, loss of CH_3OH; 354 = 474 - $CH_2C_6H_4OCH_2$ (type A); 340 = 474 - $CH_2CH_2C_6H_4OCH_2$ (type A) or 474 - $CH_3OC_6H_4CH{=}CH_2$ (type B); 284 = 474 - [O-glucuronide and CH3], or 492 - [$CH_3CH_2CH_2CH_2C_6H_4OCH_3$ and CO_2] (type A); 173 = 474 - [O-glucuronide and OCH_3 and phenyl ring +H]; 164 = $CH_3CH_2CH_2CH_2C_6H_4OCH_3$ (type A); 136 = $CH_3CH_2C_6H_4OCH_3$ (type A) or $CH_3OC_6H_4CHO$ (type B); 121 = 136 - CH_3; 107 = $CH_3OC_6H_4$.

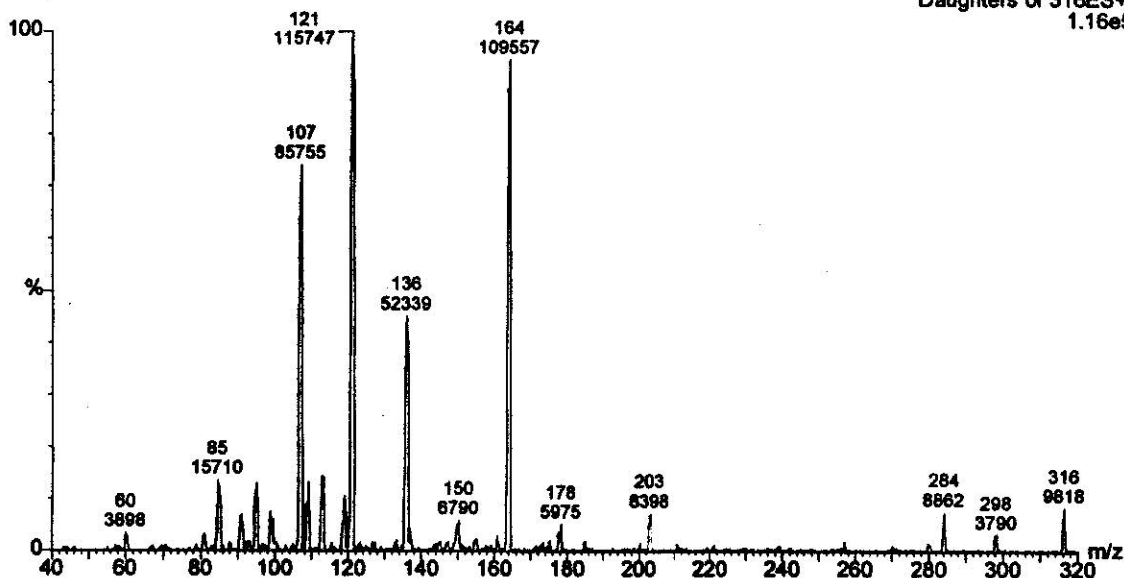
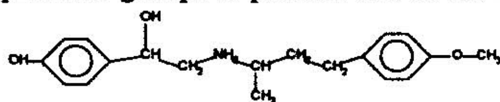
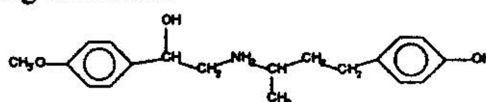


Fig. 7. Daughter ion spectrum by ESI(+) of m/z 316 ion seen in Fig. 3 [bottom]. The pattern is consistent with addition of a methyl group presumably by methylation at the phenolic groups to provide one of the following structures:

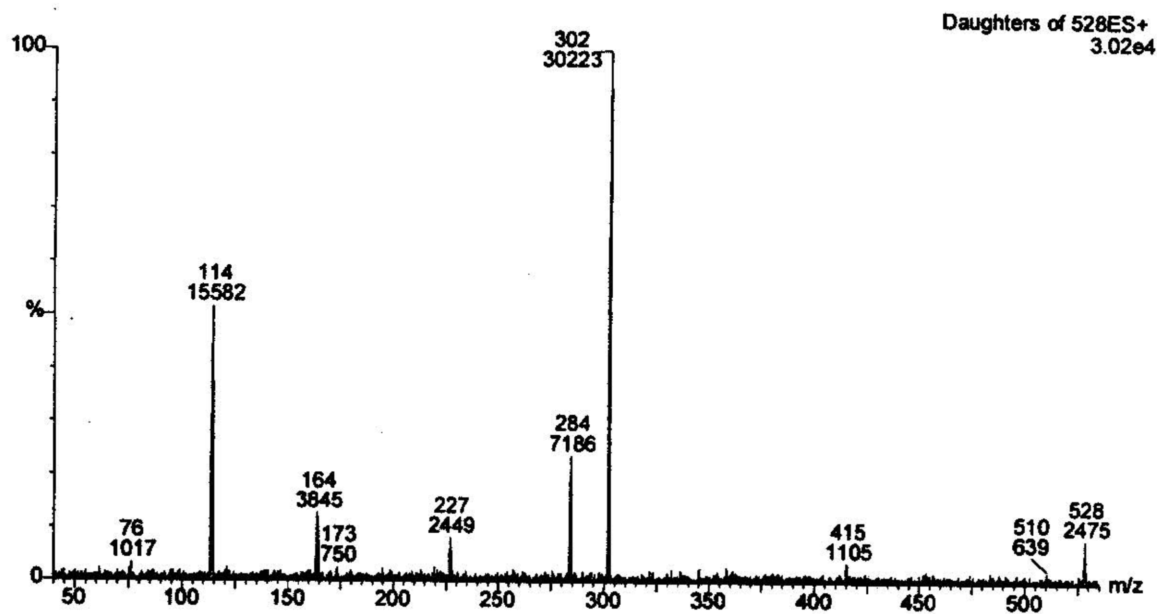


Type A



Type B

Assignments: m/z 298, loss of H_2O (benzylic alcohol); 284, loss of CH_3OH; 203, loss of $C_5H_4OCH_3$; 178, several possibilities from 316 or 298; 164 = $CH_3CH(NH)CH_2CH_2C_6H_4OH$ (type A) or $CH_3CH_2CH_2CH_2C_6H_4OCH_3$ (type B); 150= $CH_3CH_2CH_2CH_2C_6H_4OH$ (type B); 136=$CH_3CH_2C_6H_4OCH_3$ (type A); 121 = $CH_2CH_2C_6H_4OH$ (type B) or $CH_2C_6H_4OCH_3$ (type A); 107 =$HOC_6H_4CH_2$ or $C_6H_4OCH_3$; 85 = $CH_2NHCH(CH_3)CH_2CH_2$.



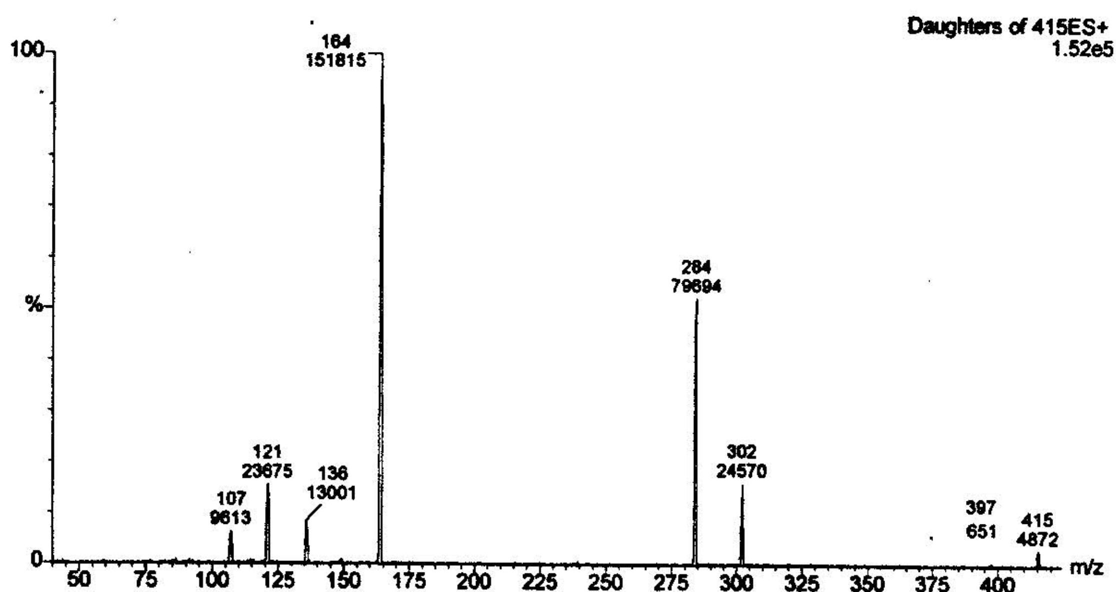


Fig. 8. Daughter ion spectrum by ESI(+) of possibly related metabolites, m/z 528 [top] and m/z 415 [bottom] as seen in Fig. 4 [top]; these metabolites apparently add 113 amu either once to make m/z 415, or twice to make m/z 528.

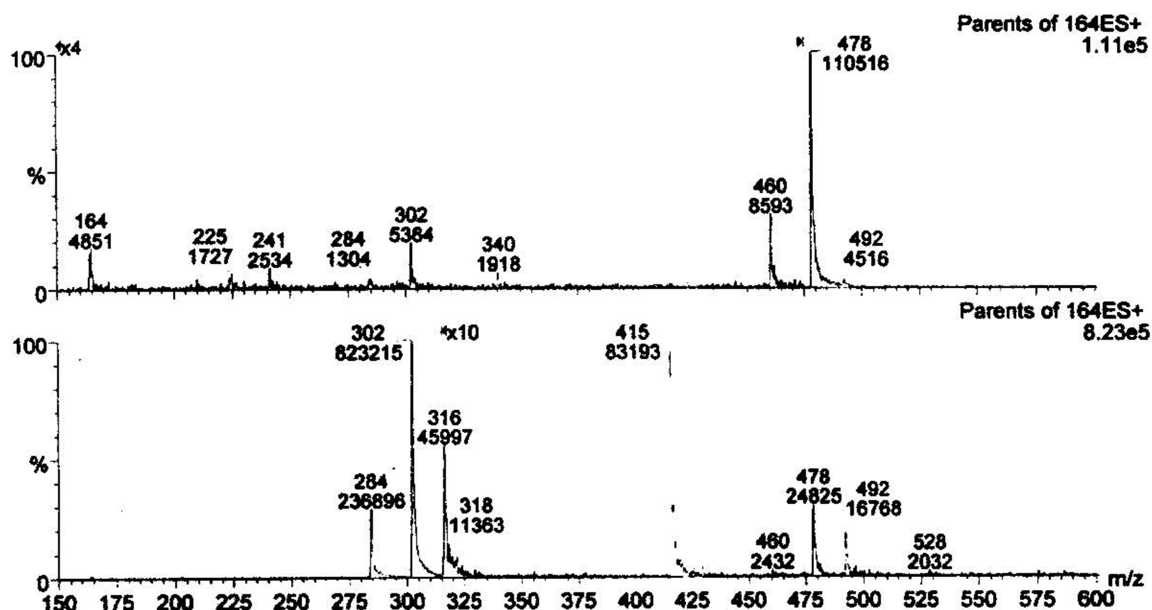


Fig. 9. Parent ion scan for the ractopamine m/z 164 NHCH(CH3)CH2CH2C6H4OH ion before [top] and after [bottom] beta-glucuronidase treatment. Metabolites (or dehydrates) thus demonstrated related to ractopamine include ions m/z 284, 302, 316, 415, 429, 478, 492, and 528.

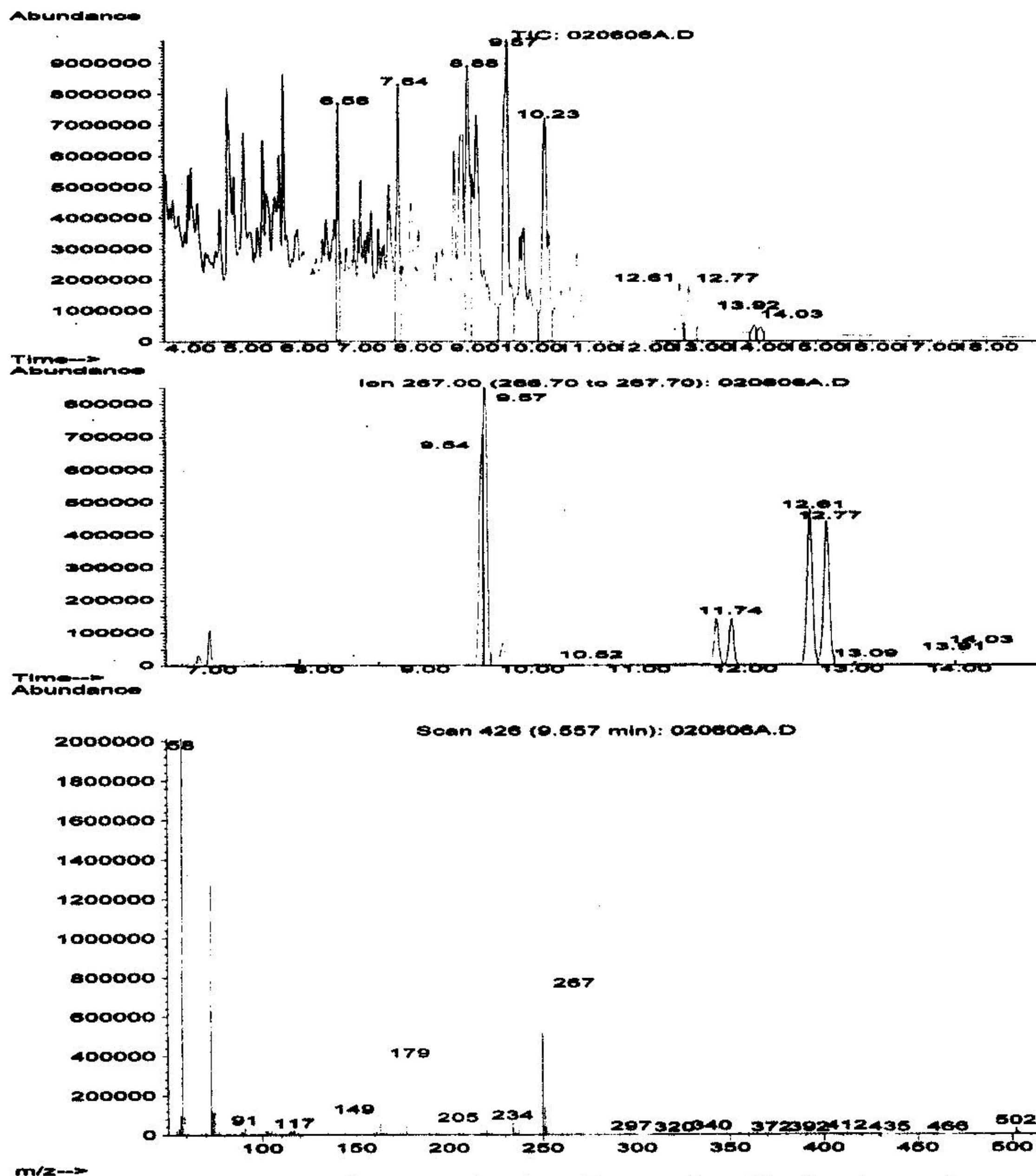


Fig. 10. GC/MS analysis of ractopamine-dosed horse urine. The four-hour urine was run as described under Materials and Methods. Top figure, total ion chromatogram. Middle figure, ion chromatogram for the significant m/z 267 ion of ractopamine. Bottom, mass spectrum of the 9.56 min peak, demonstrating a m.w. mass of 502 and providing a 99% match to library standard of ractopamine-tris(TMS).

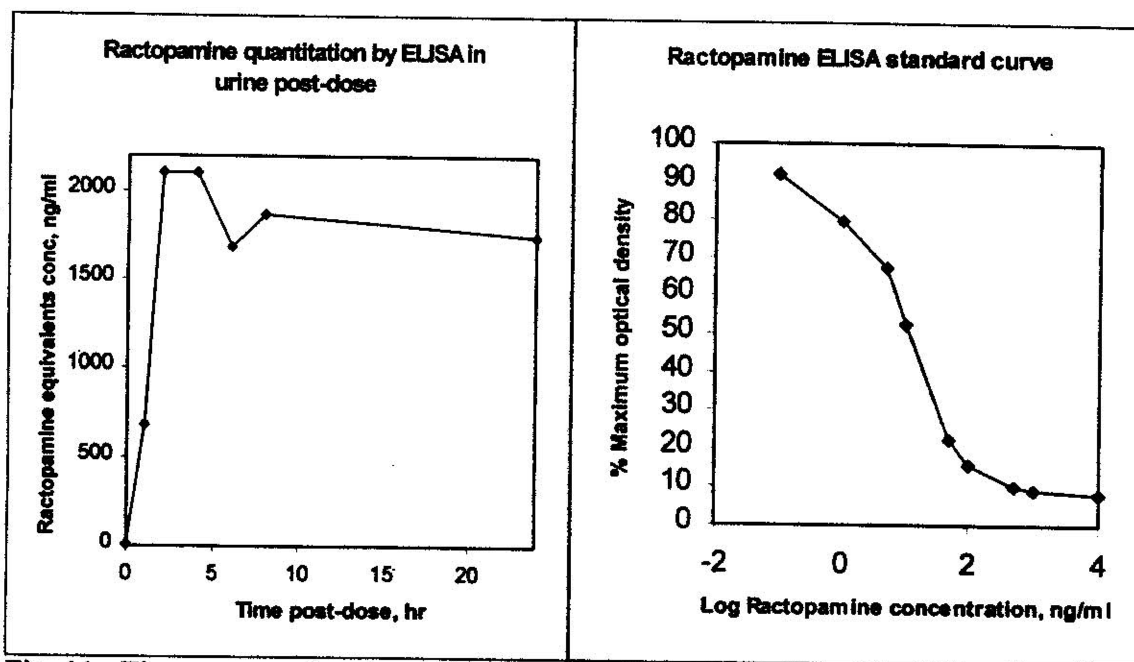


Fig. 11. Time course of ractopamine excretion in urine following 300 mg oral dose, as determined by ELISA [left]; the ractopamine ELISA standard curve [right] was developed over a five-log range in order to determine the region of linearity best suited for quantitative purposes, namely 1.0 to 100 ng/ml.

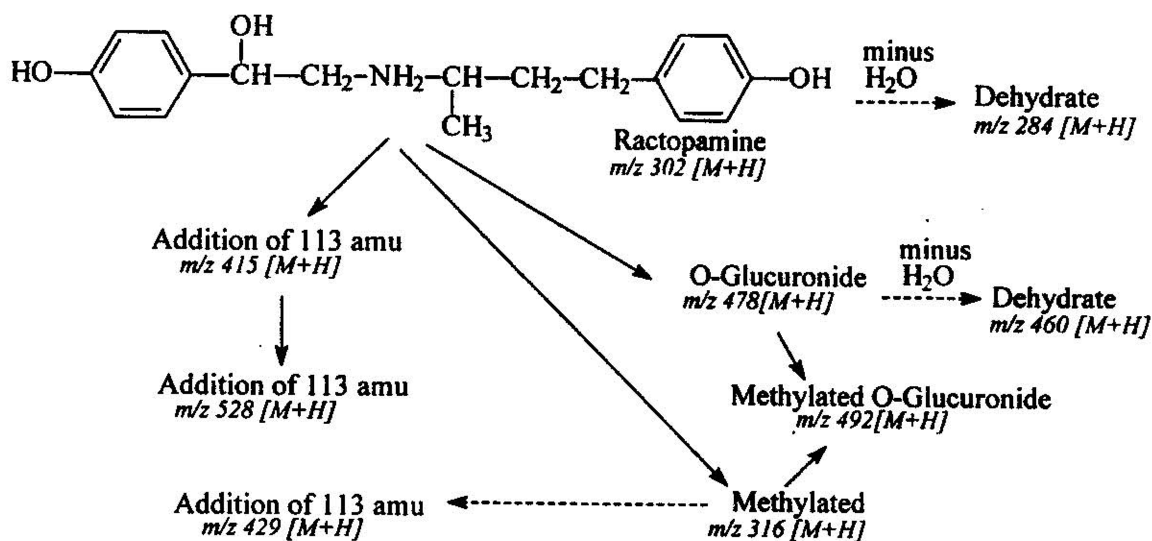


Fig. 12. Summary scheme of equine ractopamine metabolism. ESI(+)MS M+H m/z values are included with each structure class. Evidence for the combined addition of 113 amu with methylation is considered tentative at best and is indicated with a dotted line.

Table 1. Predicted molecular weights of ractopamine metabolites found during this study, and their TMS derivatives, and TMS derivatives minus a single CH3 group [-15 amu]

Compound	MW	TMS	TMS2	TMS3	TMS4	TMS5	TMS6	TMS7
Ractopamine	301	373	445	517	589			
Methylated ractopamine	315	387	459	531				
Ractopamine-O-Glucuronide	477	549	621	693	765	837	909	981
Methylated ractopamine-O-Glucuronide	491	563	635	707	779	851	923	
Unknown ractopmine metabolite+113	414	486	558	630	702			
Unknown ractopamine metabolite+113 x 2	527	599	671	743	815			
Methylated unknown ractopamine metabolite+113	428	500	572	644	716			
Loss of a single methyl group from above:								
Ractopamine	286	358	430	502	574			
Methylated ractopamine	300	372	444	516				
Ractopamine-O-Glucuronide	462	534	606	678	750	822	894	966
Methylated ractopamine-O-Glucuronide	476	548	620	692	764	836	908	
Unknown ractopmine metabolite+113	399	471	543	615	687			
Unknown ractopamine metabolite+113 x 2	512	584	656	728	800			
Methylated unknown ractopamine metabolite+113	413	485	557	629	701			

Table 2. Summary of results found by GC/MS analysis of 4-hour post-dose urine ractopamine metabolites and their likely assignments.

RT by GC/MS, min	RT of likely diastereomeric cognate, min	Highest EI mass visible, m/z	Possible ID
9.54	9.57	502	99% match to ractopamine-tris(TMS)
10.50	10.52	574	Possible ractopamine-tetrakis(TMS)
11.73	11.87	618	loss of TMS-O-C_6H_4-CH_2CH=$CHCH_3$ from ractopamine-O-glucuronide
12.61	12.76	527	loss of CH_3-O-C_6H_4-CH_2CH=$CHCH_3$ and O-TMS from methylated ractopamine-O-glucuronide$(TMS)_4$
13.10	13.31	351	loss of TMS-O-C_6H_5 from methylated ractopamine$(TMS)_3$
13.91	14.05	625	unassigned