Developed for Testing Integrity Program by Equine Pharmacology Laboratory, Gluck Equine Research Center, University of Kentucky

Charlie G. Hughes

Confirmation and Quantitation of Ractopamine in Equine Urine

I. INTRODUCTION

The use of pharmacologic agents to promote or regulate growth of animals is not uncommon. In general, these agents are, or mimic, endogenous compounds. Among the effects of many beta-adrenergic agonists is the ability to partition the nutrient supply to increase muscle growth and decrease fat deposition. Ractopamine (Paylean®) is a beta-adrenergic agonist and growth regulator approved by the FDA for use in swine.

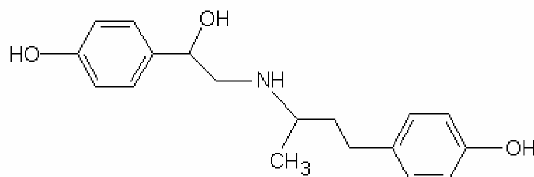


Figure 1. Ractopamine, m.w. 301

Ractopamine, (±)-*all-rac-p*-hydroxy-α-[[[3-(*p*-hydroxyphenyl)-1-methyl-propyl]amino]methyl] benzyl alcohol, consists of a racemic mixture of isomers.

Since beta-adrenergic agonists have the potential to affect the performance of racing horses a method to determine the use of ractopamine is required.

II. SCOPE

This procedure describes a method for the confirmation of ractopamine positive tests obtained from ELISA or TLC screening of equine urine. Solid phase extraction (SPE) of ractopamine is followed by derivatization and analysis by selected ion monitoring GC/MS. Ractopamine was easily identified in the urine of a horse 24 hours after oral administration of 300 mg of the drug. The assay is of sufficient sensitivity to confirm positive ELISA screening results.

III. METHOD SUMMARY

The ractopamine molecule has both a high molecular weight and polar constituents which make it unsuitable for analysis by gas chromatography. However gas chromatography may be used for its analysis after the formation of less polar derivatives. Ractopamine is extracted from hydrolyzed urine by solid phase extraction (SPE). The extract is evaporated to dryness, redissolved and derivatized in a mixture of DMF and BSTFA+1% TMCS, and injected into the GC/MS with monitoring of ions specific to ractopamine and the internal standard. A standard curve is constructed by plotting standard ractopamine concentration vs. the ratio of

ractopamine/internal standard peak areas. An estimate of ractopamine concentration in unknown samples is obtained by comparing unknown/internal standard area ratio obtained from the unknown sample with the standard curve.

IV. REAGENTS

A. Water, deionized distilled, 18 mΩ/cm^2

B. Solid Phase Extraction Solvents and Reagents
--organic solvents are HPLC grade; water is deionized distilled, reagents are analytical grade or better.

1. Methanol

2. 1M Acetic acid
To prepare, add 23 ml glacial acetic acid to 200 ml water, mix, then dilute to a total volume of 400 ml. Store at room temperature.

3. 0.1M Sodium phosphate buffer, pH 6
To prepare, add 1.36 g potassium phosphate monobasic per liter of water. Adjust to pH 6.0 with 1M NaOH. Store at room temperature.

4. Dichloromethane / isopropanol / NH_4OH (~30% (w/v)), 78:20:2 (v:v:v).
Prepare daily. Store at room temperature.

C. Derivatizing Reagent
BSTFA + 1% TMCS (Pierce Chemical Company, Product # 38831) Contents of ampoules are transferred to a screw cap vial and stored in a dessicator in a refrigerator.

D. Dimethylformamide, A.R. grade

E. Compressed Gases
Nitrogen Gas, pre-purified.
Helium, zero grade

F. Standards
Ractopamine HCl. USDA ARS.
Isoxsuprine HCl. Sigma Chemical Company R-0758

G. ß-Glucuronidase (EC 3.2.1.31) Type L-II from limpets, Sigma G-8132

H. ß-Glucuronidase stock solution.
Dilute ß-glucuronidase powder with deionized water to a concentration of 5000 units/ml and store in refrigerator for up to 2 weeks.

I. 1 M Sodium acetate buffer, adjusted to pH 5 with acetic acid. To prepare, add 136 grams NaCH3COO-(H2O)3 to 800 ml deionized water; adjust to pH 5 with 1M acetic acid; dilute to a final volume of 1000.

V. STANDARDS

A. Ractopamine stock standard

Weigh ractopamine on an analytical balance and dissolve in methanol to yield a concentration of ractopamine base of 1.00 mg/ml. Prepare this solution monthly and store in a –20°C freezer. Allow to come to room temperature before opening container and measuring aliquots.

B. Ractopamine working standard

Prepare dilutions of ractopamine stock standard with methanol to yield concentrations of 10, 1.0, 0.1 and 0.0 ng/ul in methanol. Prepare fresh daily. The stock concentration is 1 mg/ml = 10^6 ng/ml = 1000 ng/ul. In 13 x 100 mm tubes make 1/10 serial dilutions of 100 ul of standard into 900 ul methanol as follows:

Dilution	ng/ml	ng/ul
none (stock)	10^6	1000
1/10	10^5	100
1/10	10^4	**10**
1/10	10^3	**1.0**
1/10	10^2	**0.1**
no standard	0	**0.0**

These solutions will be used to prepare calibrators.

C. Isoxsuprine stock internal standard

Weigh isoxsuprine HCl on an analytical balance and dissolve in methanol to yield a concentration of isoxsuprine base of 1.00 mg/ml. Prepare this solution monthly and store in a –20°C freezer. Allow to come to room temperature before opening container and measuring aliquots.

D. Isoxsuprine working internal standard

Dilute stock internal standard with methanol to yield a working solution of 10 ng/ul in methanol. Prepare fresh daily.

VI. SUPPLIES

A. 12 ml screw cap centrifuge tubes.

B. Solid phase extraction cartridges, 3 ml, 200 mg bed, Type CSDAU (World-Wide Monitoring, Clean Screen®)

C. Glass culture tubes for collecting eluent (12 x 75 or size to fit evaporating apparatus)

D. Screwcap Agilent 7673 autosampler vials fitted with silanized 100 µl polyspring inserts (National Scientific Company)

VII. APPARATUS

A. Adjustable volume pipettors, 1-10 µl, 10-100 µl, 200-1000 µl (Eppendorf Reference) maintained and calibrated to < 2% error.

B. Vortex mixer (American Scientific Products)

C. pH meter with calibration standards (eg. stds of pH 4 and 7)

D. Nitrogen evaporator (Organomation Multivap, Zymark Turbovap, or other evaporator capable of introducing controlled N_2 flow)

E. Heating block or water bath (65°C).

F. Speedisk 48 Pressure SPE Processor, SPEware Corporation, San Pedro, CA. Also acceptable are the Zymark positive pressure SPE system or other vacuum or positive pressure SPE manifolds.

VIII. CALIBRATORS AND SAMPLE PREPARATION

A. 2 ml of each urine to be analyzed is pipetted into a 12 ml screw cap centrifuge tube.

B. In the same manner prepare 2 ml of blank urine for each calibrator or standard.

C. Hydrolysis -- add to each tube 800 ul 1M sodium acetate, pH 5 and 400 ul of 5000 U/ml ß-glucuronidase solution. Cap the tubes, vortex, then incubate at 65°C for 3 hours.

D. Cool the tubes, then spike all tubes with 100 ul of isoxsuprine internal standard solution.

E. Spike calibrator tubes with ractopamine working standard to obtain calibrators in the range of 0 - 500 ng/ml as follows:

Preparation of Calibrators

Standard spike concentration ng/ul	Spike volume ul	Calibrator final conc ng/2 ml	Calibrator final conc ng/ml
0	50	0	0
0.1	100	10	5
1.0	50	50	25
1.0	100	100	50
10	30	300	150
10	50	500	250
10	100	1000	500

F. Mix tubes by vortex (2-3 seconds), then centrifuge 10 min at 2000 rpm to eliminate particulate. Subject supernatant to solid phase extraction.

IX. SOLID PHASE EXTRACTION AND DERIVATIZATION

A. The Speedisk 48 SPE Processor system was used to develop this assay. The procedure is adapatable to other systems, such as the Zymark RapidTrace® automated system or any manual vacuum or positive pressure manifold. (See Appendix I for instrumental set-up for Zymark RapidTrace® extraction)

B. Condition SPE columns by adding sequentially 3 ml methanol, 3 ml water, 1 ml 0.1M sodium phosphate buffer, pH 6.0. Do not allow column to dry between additions.

C. Load samples at a rate of 1 ml per minute, then wash the column sequentially with 2ml water, 2 ml 1M acetic acid, 4 ml methanol.

D. Dry column with N_2 or air flow for 1 minute.

E. Elute column with 3 ml dichloromethane/isopropanol/NH_4OH (78:20:2). Collect eluent in glass evaporator tubes.

F. Evaporate eluent to dryness under a stream of N_2 in a 38-40°C water bath.

G. Dissolve residue in 15 µL of DMF plus 60 µL of BSTFA-1% TMCS. Derivatization occurs readily upon dissolving. Immediately transfer this solution to a micro injection vial, seal with a septum cap, and load the GC autosampler tray.

H. Inject 1 µL into GC/MS via autosampler.

X. GAS CHROMATOGRAPHY -- MASS SPECTROSCOPY

A. GC column: HP-5 MS, 30 m x 0.25 mm x 0.25 µm film thickness operated in the splitless mode.

B. The GC oven temperature is programmed as follows: 180°C for 2 min, then increased to 280°C at 20°C/min, and held at 280°C for 5 minutes. The injector temperature is maintained at 250°C

C. The mass spectrometer (HP 6890/5972 GC/MSD) is tuned with PFTBA using the manufacturer's standard autotune procedure.

D. Data is collected using selected ion monitoring (SIM) during the chromatographic runs.

E. The 178 m/z ion of bis(TMS)-isoxsuprine derivative is the major fragmentation product and is monitored as the internal standard ion.

F. Ions monitored for the tris(TMS)-ractopamine derivative are 267, 250, and 179 m/z. The origin of these major ions may be explained by scission of the molecule as indicated in Figure 2, with the 502 m/z ion resulting from loss of CH_3 from the molecular ion, M^+, m/z 517, which is not observed.

Table 2. SIM Dwell Times for ractopamine and isoxsuprine TMS derivative ions.

m/z	Dwell (msec)
ractopamine	
502.2	50
267.2	50
250.2	50
179.1	50
isoxsuprine	
178.1	20

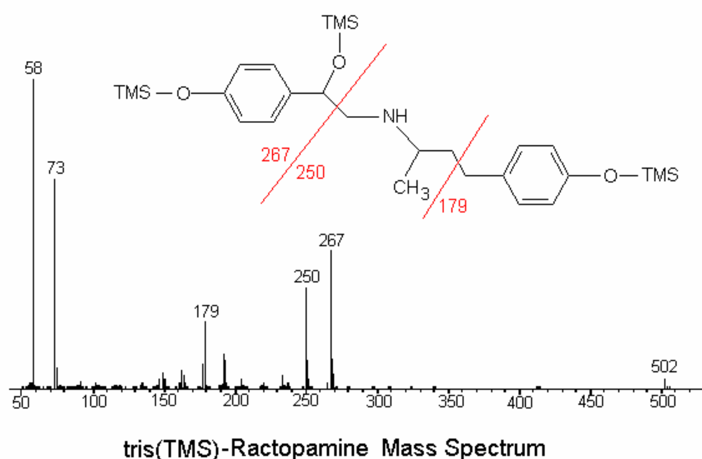
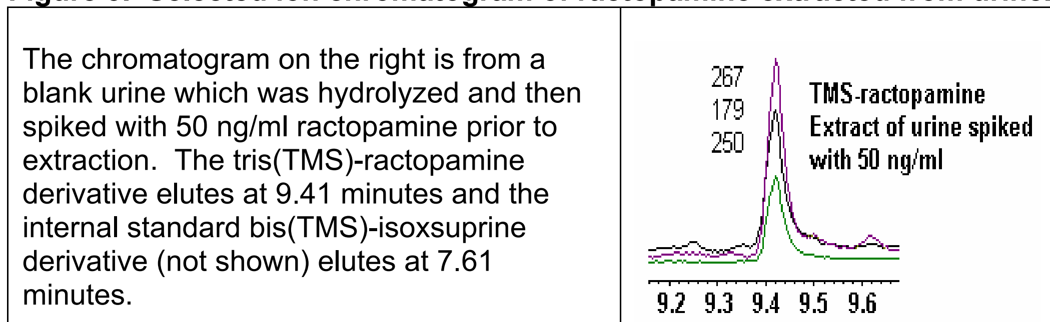


Figure 2. Mass Spectrum of Ractopamine Derivative

G. Three major ractopamine ions were selected as diagnostic ions for ractopamine were 179, 250, and 267 m/z. Quantitation was based on the most abundant ion, 267 m/z.

Figure 3. Selected ion chromatogram of ractopamine extracted from urine.



The chromatogram on the right is from a blank urine which was hydrolyzed and then spiked with 50 ng/ml ractopamine prior to extraction. The tris(TMS)-ractopamine derivative elutes at 9.41 minutes and the internal standard bis(TMS)-isoxsuprine derivative (not shown) elutes at 7.61 minutes.

H. Using this procedure ractopamine was easily identified in hydrolyzed equine urine 24 hours after oral administration of 300 mg of the drug.

XI. RACTOPAMINE STANDARD CURVE

A. Preparation of the standard curve is accomplished by determining the internal standard (178 m/z) and ractopamine (267 m/z) peak areas and plotting concentration on the vertical axis vs. the ratio of ractopamine/internal standard area on the horizontal axis.

B. The standard curve shows a linear response up to 100 ng/ml with a correlation coefficient R > .99.

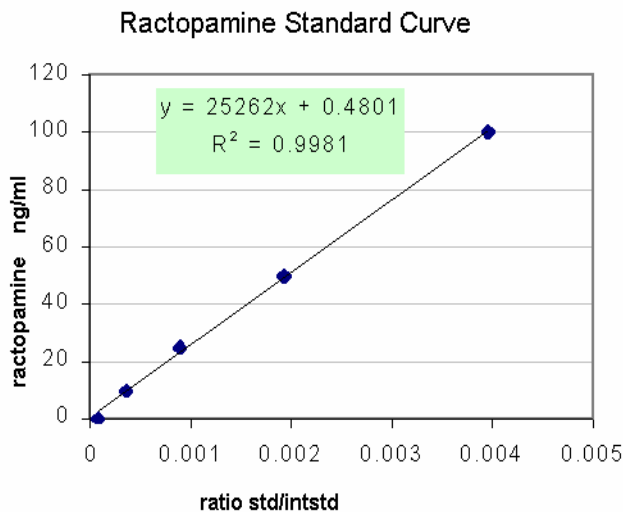


Figure 4. Typical Standard Curve

Ractopamine standard curves over a narrow range of concentrations such as 0 – 100 were linear as shown in Figure 4. Curves over a concentration range 0 – 1000 ng/ml usually exhibited a non-linear response and required use of second order regression to perform adequate quantitations of unknowns. Use of the narrow range standard curve with appropriate dilution of unknowns where necessary is the preferred approach to analysis of samples having high concentrations.

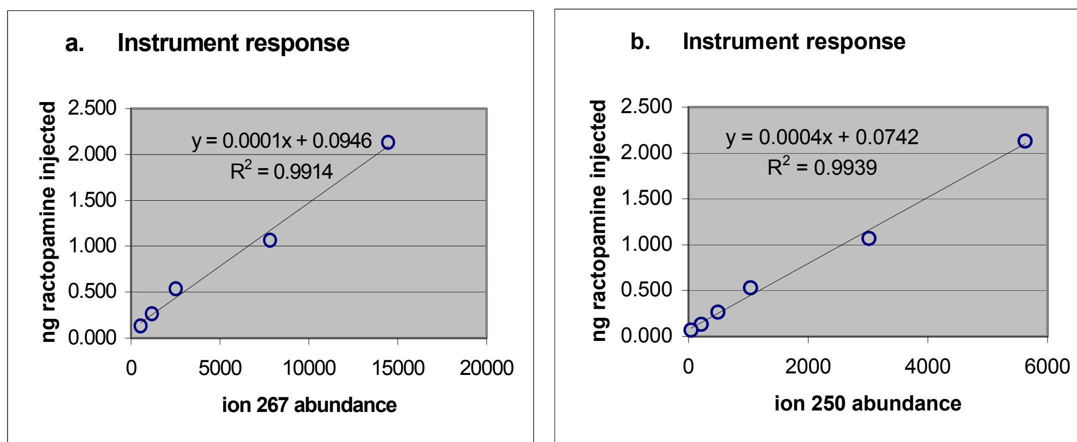
XII. VALIDATION OF ASSAY

A. Instrument Linearity
A series of injections of decreasing concentrations of derivatized TMS-ractopamine as shown in Table 3 was made to verify instrument linearity of response. As shown in Figures 5a and 5b, plots of ion abundance vs. concentration produced a linear response for TMS-ractopamine ions 250 and 267 at quantities injected between 2.1 and 0.1 ng with a regression coefficient greater than 0.99.

Table 3. Ion Abundance Ratios for TMS-Ractopamine

SIM chromatographic peak areas Quantities are weight of ractopamine which was derivatized to form TMS-ractopamine.					**AORC ion ratio criteria ± 30% of relative abundance**		
					49.0--91.0	**27.6--51.4**	**100.0**
ng/75uL	ng inject	179 m/z	250 m/z	267 m/z	179 m/z	250 m/z	267 m/z
160	2.13	10065	5621	14468	69.6	38.9	100.0
80	1.07	5286	3020	7814	67.6	38.6	100.0
40	0.53	1830	1033	2517	72.7	41.0	100.0
20	0.27	913	491	1191	76.7	41.2	100.0
10	0.13	368	212	534	68.9	39.7	100.0
5	0.07	234	48	206	113.6	23.3	100.0

Figure 5. Instrument Linearity for two tris(TMS)-ractopamine ions, m/z 267 (a) and m/z 250 (b)



B. Identity Confirmation of Non-extracted Standards
The proposed AORC criteria for identity confirmation (Appendix II) for chromatographic and mass spectral data consisting of at least 3 diagnostic ions were applied to the data in Table 3. The on-column quantity of tris(TMS)-ractopamine met these criteria down to approximately 0.1 ng (values exceeding these limits are shaded gray)

C. Instrument Lower Limit of Detection
Table 4 shows the signal to noise ratio (S/N) for the major tris(TMS)-ractopamine ions as on-column quantities of tris(TMS)-ractopamine are decreased. S/N falls below 3 at approximately 0.1 ng injected. Values failing the S/N ≥ 3 criterion are shaded in gray.

Table 4. Instrument Lower Limit of Detection

Signal to noise ratio (S/N) for specific ions at different concentrations calculated from SIM chromatographic peak areas. Detection limit defined as S/N = 3				
ng/75uL	ng injected	S/N 179 m/z	S/N 250 m/z	S/N 267 m/z
20	0.27	13.7	12.5	8.5
10	0.13	4.4	5.9	3.6
5	0.07	1.6	2.5	2.5

D. Extraction efficiency of the SPE columns for ractopamine ranged between 40-50%.

E. Lower Limit of Detection for Ractopamine in Urine
Selected ion chromatograms for urine samples spiked with ractopamine showed measurable chromatographic peaks for TMS-ractopamine down to about 10 ng/ml when 2 ml samples were extracted. However most samples failed the AORC criteria for confirmation of identity at urine ractopamine concentrations of approximately 25 ng/ml or less. All of 28 samples spiked with 50 ng/ml met the AORC ion ratio criteria for confirmation. This level of sensitivity compares favorably with the ELISA screening detection limit of 50 ng/ml.

F. Recovery of ractopamine spiked into urine at concentrations of 50 and 500 ng/ml on four different days was measured.

Table 5. Recovery of Ractopamine

50 ng/ml ± c.v.	500 ng/ml ± c.v.
44.3 ± 9.6%	484 ± 6.6%
48.8 ± 8.9%	499 ± 2.7%
40.7 ± 20%	572 ± 15%
45.9 ± 23%	474 ± 12%

It is reasonable to expect both the precision and accuracy of this assay to be improved through the use of a ractopamine isotopic internal standard should it become available.

G. Freeze/Thaw Stability --
40 ml urine was spiked with 10 ug ractopamine in 100 ul methanol to produce a concentration of 250 ng/ml in urine. The urine was mixed thoroughly and poured into eight test tubes to make four duplicate samples which were placed in the freezer.
day 1 -- tubes 4a and 4b thawed and refrozen
day 2 – tubes 4a,4b,3a,3b thawed and refrozen
day 3 – tubes 4a,4b,3a,3b,2a,2b thawed and refrozen
day 7 – all tubes thawed and analyzed for ractopamine
All samples were above 90% of spiked values with a mean of 96.2%.

H. Stability of Ractopamine Silyl Derivative –
Gas chromatography of the tris(TMS)-ractopamine must proceed promptly after formation of the derivative. The internal standard isoxsuprine derivative is stable at room temperature, however the ractopamine derivative decreases to about half its original concentration after storage overnight (about 16 hours) at room temperature. For extended analytical runs calibrators must be run both before and after the unknown to insure accuracy. Problems with errors in quantitation could be eliminated or lessened by the use of a deuterated ractopamine analog internal standard.

XIII. REFERENCES

AORC. Minimum criteria for identification by chromatography and mass spectrometry. Association of Official Racing Chemists Proposed Guidelines, 2001. Available from AORC, Lexington, KY.

Testing Components Corp. (TCC) Ractopamine ELISA screening test kit protocol.

Lehner AF, Hughes CG, Harkins JD, et al. Metabolism of the beta-agonist ractopamine in the horse. (in preparation) 2002

Lehner AF, Hughes CG, Harkins JD, et al. Detection and confirmation of the ß-agonist ractopamine and its metabolites in equine urine. (in preparation for J. Anal. Toxicol) 2002

APPENDIX I – Zymark Solid Phase Extraction Sequence

	Step	Source	Output	Vol	ml/min	Liquid Sense
1	Condition	MeOH	Org/Aq	3	20	No
2	Condition	dH2O	Aq	3	20	No
3	Condition	NaPO4	Aq	1	20	No
4	Load	Sample	Aq	4	1.5	No
5	Rinse	dH2O	Aq	2	20	No
6	Purge Cannula	dH2O	Cannula	5	20	No
7	Rinse	Acetic	Aq	2	20	No
8	Rinse	MeOH	Org/Aq	4	20	No
9	Purge Cannula	dH2O	Cannula	1	20	No
10	Dry	- - -	Time =	2	min	No
11	Purge Cannula	MeOH	Cannula	5	20	No
12	Collect	DCM+	Fract 1	3	1.5	No
13	Purge Cannula	dH2O	Cannula	3	20	No

APPENDIX II -- Summary of AORC Criteria for Identification by Chromatographic methods coupled with Mass Spectrometry (AORC, 2001)

Chromatography--
Unknown and standard retention times must be in agreement.

Mass Spectrometry--
-- A minimum number of three specified, diagnostic ions should be obtained for any technique. A diagnostic ion is defined as a molecular ion or fragment ion whose presence and abundance are characteristic of the analyte.
-- The molecular ion should be included among the diagnostic ions if it is present at a relative abundance greater than 5% of the base peak.
-- The signal to noise ratio of the diagnostic ions must be equal to or greater than 3:1, measured by integrated single mass traces (single ion chromatograms).
-- Measured diagnostic ions with a relative intensity >10% of base peak in the reference spectrum must be present in the test spectrum.

<u>Maximum permitted difference (tolerance) for matching diagnostic ions.</u>

The relative abundance (intensity) is the abundance of a particular ion relative to the most abundant diagnostic ion monitored expressed as a percentage.

Relative intensities are calculated by integrating the signals of single ion chromatograms.

The maximum permitted differences in relative abundance should be as follows:
-- Low resolution MS or SIM: The greater of 5% absolute or 30% relative.
-- MS-MS and related techniques: The greater of 15% absolute or 35% relative.

The maximum permitted difference (tolerance) in relative abundance may be absolute or relative.
-- An absolute tolerance range of 5% is defined as the standard ion's relative abundance ± 5. For example, the acceptable absolute range for a relative abundance of 56% would be 56 ± 5, or a range of 51%-61%.
-- A relative tolerance of range of 30% is defined as the standard ion's relative abundance ± 30% of that abundance. For example, the acceptable relative tolerance range for a relative abundance of 60% would be 60 ± (30% of 60), or a range of 42%-78%.

Extraneous ions in the test spectrum should not exceed 15% relative abundance.

More rigorous criteria may be appropriate for SIM data; if possible, a minimum of 4 diagnostic ions or stricter limits on relative intensities.