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Title: Determination of Ractopamine Hydrochloride by High Performance Liquid Chromatography		
Revision: .02	Replaces: CLG-RAC1.01	Effective: 05/03/2013

Contents

A. INTRODUCTION

1. Summary of Procedure

Residual ractopamine, a β-adrenergic agonist, is extracted from swine and bovine liver and muscle with methanol. An aliquot of the extract is evaporated, borate buffer is added, and ractopamine is extracted into ethyl acetate by liquid/liquid partition. The ethyl acetate extract is further purified by passing it through an acidic alumina solid phase extraction column. Ractopamine is eluted from the column with methanol. The methanol extract is evaporated to dryness, then dissolved in either dilute acetic acid or a 1:1 mixture of dilute acetic acid and methanol, depending upon the tissue extracted. The extract is filtered and analyzed for ractopamine using high performance liquid chromatography (HPLC) with fluorescence detection. The residual ractopamine is quantified as ractopamine hydrochloride (HCl). Ractopamine is a mixture of four stereoisomers in approximately equal proportions (RS, SR, RR, and SS). This HPLC method does not distinguish between these stereoisomers, and thus results in a single peak for all four stereoisomers present in a particular sample. The method does not use enzymatic hydrolysis to quantify conjugated ractopamine metabolites.

2. Applicability

This method is suitable for the quantification of ractopamine HCl in swine and bovine liver at 25 - 300 ppb and in swine and bovine muscle tissues at 1.0 - 300 ppb.

Note: Refer to 21CFR for tolerance values set by FDA and 40CFR for tolerance values set by EPA.

B. EQUIPMENT

Note: Equivalent equipment may be substituted.

1. Apparatus

a. Analytical balance (± 0.0001 g) - AG204, Mettler Toledo.

b. Top loading balance (± 0.01 g) - PG5002.S Delta Range, Mettler Toledo.

c. Magnetic stirrer and stir bars Corning Stirring/Hot Plate - PC 420, and VWR, 5/16” Diameter x 2” stir bars, 58949-038.

d. Meat grinder or food processor - model 2100 M/2, Rival.

e. Branson Sonifier 450 ultrasonic generator with a 1/4 inch micro tip or a mechanical blender such as an UltraTurrax No.T25 equipped with an S25N-10G dispersing rotor.

f. Vortex-2 Genie test tube vortexer - Scientific Industries, Bohemia, NY.

g. Polypropylene centrifuge tubes - 50 mL conical, with closures, Blue Max, Cat. No. 352098, 30 x 115 mm, 25/rack, VWR.

h. Polypropylene tubes – 15 mL conical, with closures, Blue Max, Cat. No. 352097, 17 x 120 mm, 50/rack, VWR.

i. Centrifuge - IEC Centra-8R centrifuge.

j. Volumetric flasks - 50 and100 mL volumetric flasks.

k. Micropipettes - 500 - 3000 µL.

l. Test tubes - 16 x 100 mm test tubes with closures - CMS, Inc., Cat. Nos. 339-309 and 270-671, respectively (Fisherbrand Disposable Culture Tubes, Borosilicate glass, 16 x 125 mm, catalog #14-961-30).

m. Volumetric pipettes - 10 mL and 15 mL, Class A.

n. Serological pipettes - 10 mL, disposable, polystyrene, Falcon 357530.

o. Mixing cylinders - 100 mL graduated mixing cylinders, stoppered, CMS, Inc., Cat. No. 101-527.

p. Glass bottles - 1000 mL glass bottles.

q. Pasteur pipettes - Disposable glass Pasteur pipettes.

r. Membrane filters - Disposable 13 mm PVDF Membrane 0.22 µm filters, HPLC Certified Minispike Outlet, Gelman Acrodisc PM4450T.

s. Small disposable syringes - Becton-Dickerson, 1 mL syringe, 309602.

t. Spatulas - Metal or Teflon coated.

u. N-Evap air/nitrogen stream evaporator - Meyer N-Evap Analytical Evaporator, Organomation Associates, Inc. South Berlin, MA.

v. Vacuum apparatus for solid phase cartridges, or syringes for sample application to cartridges - Supelco Visiprep, Supelco, 595 North Harrison Road, Bellefonte, PA.

w. Ultrasonic water bath - Branson model 2200, 125 watts.

x. pH meter - Orion 611, ThermoOrion, 500 Cummings Center, Beverly, MA.

y. Solid Phase Extraction (SPE) Cartridges - Acidic alumina (Alumina A) approximately 2 g packing, Activity Grade I, Waters Sep-Pak Classic, No. 51800, Waters Sep-Pak Plus. No. 20500.

Note: Not all brands of acidic alumina produce acceptable results. See, Section K.1, Appendix, SPE CARTRIDGE TESTING.

2. Instrumentation

   a. HPLC pump - Agilent 1100 series quaternary pump, G1311A.

   b. HPLC autosampler - Agilent 1100 series ALS, G1313A.

   c. HPLC variable wavelength fluorescence detector - Agilent FLD, G1321A.

   d. HPLC detector recording device - Agilent Chem Station, Agilent Technologies, Wilmington, Delaware.

   e. HPLC Column - C18 reversed phase such as Supelcosil LC-18-DB, 5 µm, 4.6 x 250 mm, Part No. 58355.

   f. HPLC mobile phase filtering and degassing apparatus - Millipore Microfiltration Assembly, 47 mm, filtering with a type GV 0.2 micron filter.

C. REAGENTS AND SOLUTIONS

Note: Equivalent reagents / solutions may be substituted. The stability time frame of the solution is dependant on the expiration date of the components used or the listed expiration date, whichever is soonest.

1. Reagents

   a. Water (H_2O) - HPLC grade or distilled, deionized.

   b. Methanol (MeOH) - HPLC grade, A452-4, Fisher.

   c. Acetonitrile (ACN) - HPLC grade, A998-4, Fisher.

   d. Ethyl acetate - HPLC grade, E195-4, Fisher.

   e. Glacial acetic acid - Reagent grade, A38-212, Fisher.

   f. Sodium hydroxide (NaOH) - Reagent grade, S318-1, Fisher.

   g. 1-Pentanesulfonic acid sodium salt - HPLC grade, 2841-05, Baker.

   h. Sodium borate decahydrate - Reagent grade, 3570-01, Baker.

2. Solutions

   a. 1 N sodium hydroxide solution:

      Dissolve 40 g of sodium hydroxide in approximately 800 mL of deionized water. Mix well. Cool to room temperature and dilute to 1 L. Store in a plastic container at room temperature. This solution is stable for one year.

      Note: Exothermic reaction. Recommend the flask be placed in an ice bath when initially dissolving the sodium hydroxide.

b. Borate Buffer (0.025M, pH 10.3):

Add 9.54 ± 0.05 g of sodium borate decahydrate to 900 mL of HPLC grade water in a graduated cylinder or glass bottle, and dissolve by mixing, add 1 N sodium hydroxide (approximately 40 mL) until the pH is 10.3 ± 0.1. Dilute to 1 L with HPLC grade water. This solution is stable for one year when stored at room temperature.

Note: Check buffer monthly. Acceptable pH range is 9.5 -11.0. There should also be no visual signs of microbial growth.

c. HPLC Mobile Phase:

Add 320 mL of HPLC grade acetonitrile to 680 mL of HPLC grade water. Then add 20 mL of glacial acetic acid and 0.87 ± 0.05 g of 1-pentanesulfonic acid sodium salt. Mix well, filter through a 0.45 µm filter if necessary, and degas. This solution is stable for one year when stored at room temperature.

d. Sample Diluent (acetic acid - 2 percent v/v):

Add 20 mL of reagent grade glacial acetic acid to 980 mL of HPLC grade water and mix well. This solution is stable for one year when stored at room temperature.

e. Sample Diluent plus Methanol Mixture (1:1 v/v):

Mix equal volumes of sample diluent and methanol in an appropriately sized storage container. This solution is stable for one year from date of preparation.

f. Mobile phase, for alternative HPLC column:

Prepare as directed in section C.2.c., except use 250 mL of acetonitrile and 750 mL of water.

D. STANDARD(S)

Note: Equivalent standards / solutions may be substituted. Purity is to be taken into account when calculating standard concentrations. The stability time frame of the solution is dependant on the expiration date of the components used or the listed expiration date, whichever ends sooner.

1. Standard Information

a. Ractopamine HCl Reference Standard - Elanco Animal Health.

b. Ritodrine HCl Reference Standard – Cat. No. 1604701, US Pharmacopeia Convention.

2. Preparation of Standard Solution(s)

   a. Prepare the following standard solutions for the analysis of liver at 25 - 300 ppb:

      i. Ractopamine HCl liver stock solution - 1.00 mg/mL

         Note: The stock solution must be adjusted for purity during preparation. Do not correct for the HCl since ractopamine HCl is the analyte of interest.

         Prepare a ractopamine HCl standard stock solution by adding 100 ± 1 mg of ractopamine HCl reference standard to a 100 mL volumetric flask and diluting to volume with the 1:1 sample diluent plus methanol mixture. This solution is stable for three months at 2 - 8 °C.

         CAUTION: Wear gloves when handling reference standard. Do not inhale the dust of the primary reference standard.

      ii. Ractopamine HCl intermediate liver standard (10 µg/mL):

         Pipet 1.0 mL of 1.00 mg/mL ractopamine HCl standard stock solution into a 100 mL volumetric flask and dilute to volume with the 1:1 sample diluent plus methanol mixture. Mix well. This solution is stable for one month at 2 - 8 °C.

      iii. Ractopamine HCl fortification liver standard (1.5 µg/mL):

         Pipet 15 mL of 10 µg/mL ractopamine HCl intermediate standard solution into a 100 mL volumetric flask and dilute to volume with the 1:1 sample diluent plus methanol mixture. Mix well. This solution is stable for one month at 2 - 8 °C.

   b. Prepare the following standard solutions for the analysis of muscle at 0.5 - 2.0 ppb:

      i. Ractopamine HCl muscle stock solution - 1.00 mg/mL

         Note: The stock solution must be adjusted for purity during preparation.

         Prepare a ractopamine hydrochloride standard stock solution by adding 100 ± 1 mg of ractopamine hydrochloride reference standard to a 100 mL volumetric flask and diluting to volume with methanol. This solution is stable for three months at 2 - 8 °C.

         CAUTION: Wear gloves when handling reference standard. Do not inhale the dust of the primary reference standard.

      ii. Ractopamine HCl Intermediate muscle standard (10 µg/mL):

         Pipet 1.0 mL of standard stock solution into a 100 mL volumetric flask and dilute to volume with sample diluent. Mix well. This solution is stable for one month at 2 - 8 °C.

iii. Ractopamine HCl intermediate muscle standard (100 ng/mL):

Pipet 1.0 mL of 10 µg/mL ractopamine HCl intermediate standard solution into a 100 mL volumetric flask and dilute to volume with sample diluent. Mix well. This solution is stable for one month at 2 - 8 °C.

iv. Ractopamine HCl fortification muscle standard (10 ng/mL):

Pipet 10 mL of 100 ng/mL ractopamine HCl intermediate standard solution into a 100 mL volumetric flask and dilute to volume with sample diluent. Mix well. This solution is stable for one month at 2 - 8 °C.

3. Resolution Standard Preparation:

Note: Prepare these solutions as necessary to check column resolution.

a. For the analysis of liver at 25 - 300 ppb.

i. Ritrodrine HCl liver stock solution (1 mg/mL):

Weigh 50 ± 0.1 mg of ritodrine HCl reference standard into a 50 mL volumetric flask and dilute to volume with the 1:1 sample diluent plus methanol mixture. This solution is stable for three months at 2 - 8 °C.

ii. Ritrodrine HCl intermediate liver standard (10 µg/mL):

Pipet 1.0 mL of 1 mg/mL ritrodrine HCl standard stock solution into a 100 mL flask and dilute to volume with the 1:1 sample diluent plus methanol mixture. Mix well. This solution is stable for one month at 2 - 8 °C.

iii. Liver resolution solution, mixed external standard (25 ng/mL):

Pipet 250 µL of the 10 µg/mL ritodrine HCl intermediate solution and 250 µL of the 10 µg/mL ractopamine HCl intermediate solution into a 100 mL flask and dilute to volume with the 1:1 sample diluent plus methanol mixture. Mix well. This solution is stable for one month at 2 - 8 °C.

b. For the analysis of muscle at 0.5 - 2.0 ppb:

i. Ritrodrine HCl muscle stock solution (1 mg/mL):

Weigh 50 ± 0.1 mg of ritodrine hydrochloride reference standard into a 50 mL volumetric flask and dilute to volume with methanol.

ii. Ritrodrine HCl intermediate muscle standard (10 µg/mL):

Pipet 1.0 mL of standard stock solution into a 100 mL flask and dilute to volume with sample diluent. Mix well.

iii. Ritodrine HCl intermediate muscle standard (100 ng/mL):

Pipet 1 mL of 10 µg/mL ritodrine HCl intermediate standard solution into a 100 mL volumetric flask and dilute to volume with sample diluent. Mix well. This solution is stable for one month at 2 - 8 °C.

iv. Muscle Resolution solution, mixed external standard (1 ng/mL):

Pipet 1 mL of the 100 ng/mL ritodrine HCl intermediate solution and 1 mL of the 100 ng/mL ractopamine HCl intermediate solution into a 100 mL flask and dilute to volume with sample diluent. Mix well. This solution is stable for one month at 2 - 8 °C.

4. Preparation of External Calibration Curve

a. Prepare the following standard solutions for the analysis of liver at 25 - 300 ppb:

i. Ractopamine HCl liver external standard curve solutions (25, 50, 75, 150 and 300 ng/mL):

Prepare volumetric dilutions of the 10 µg/mL ractopamine HCl intermediate standard using the 1:1 sample diluent plus methanol mixture. For 25, 50, 75, 150 and 300 ng/mL solutions make 250 µL to 100 mL, 500 µL to 100 mL, 750 µL to 100 mL, 1.5 mL to 100 mL and 3.0 mL to 100 mL volumetric dilutions, respectively. These solutions are stable for one month at 2 - 8 °C.

b. Prepare the following standard solutions for the analysis of muscle at 0.5 - 2.0 ppb:

i. Ractopamine HCl muscle external standard curve solutions (0.5, 0.75, 1.0, 1.5, 2.0 ng/mL):

Prepare volumetric dilutions of the 100 ng/mL ractopamine HCl intermediate standard using sample diluent. For 0.5, 0.75, 1.0, 1.5, and 2.0 ng/mL solutions make 500 µL to 100 mL, 750 µL to 100 mL, 1000 µL to 100 mL, 1500 µL to 100 mL, and 2000 µL to 100 mL volumetric dilutions, respectively. These solutions are stable for one month when maintained at 2 - 8 °C.

E. SAMPLE PREPARATION

1. Preparation and Storage of Tissues

a. Initial processing includes grinding or blending of the tissues using a food grinder (or cryogenic grinding) to produce homogenous samples. Grind a minimum 500 g sample of tissue when possible.

b. Store all tissues at < -10 °C when not processing or sub-sampling. Ractopamine has been shown to be stable in frozen tissue for one year.

Note: Extreme care should be taken to make sure all tissue residue containing ractopamine is cleaned from glassware and other laboratory items in contact with samples and standards. It is recommended that disposable items be used

whenever possible and that labware used with standards and other sources containing high levels of ractopamine be kept separate from that used to prepare samples in the low ppb range.

F. ANALYTICAL PROCEDURE

1. Preparation of Controls

   a. For liver

      i. Prepare blank and recovery samples by weighing two 10 ± 0.2 g blank tissues as part of the sample set.

      ii. For the analysis of liver at 25 - 300 ppb, prepare a 150 ppb recovery by adding 1 mL of 1.5 µg/mL fortification standard (D.2.a.iii) to one of the tissue blanks. Continue at step F.2.b.

   b. For muscle

      i. Prepare blank and recovery samples by weighing two 10 ± 0.2 g blank tissues as part of the sample set.

      ii. For the analysis of muscle at 0.5 - 2.0 ppb, prepare a 1 ppb recovery by adding 1 mL of the 10 ng/mL fortification standard (D.2.b.iii) to one of the tissue blanks. Continue at step F.2.b.

2. Extraction Procedure

   a. Tissue Extraction

      Weigh 10.0 ± 0.2 g of frozen or partially thawed ground sample tissue into a suitable container such as a 50 mL polypropylene centrifuge tube.

   b. Add 20 ± 1 mL of methanol to the sample.

   c. Homogenize the tissue slurry for approximately one minute using an ultrasonic cell disrupter equipped with a 1/4 inch micro tip. Alternatively, the tissue may be blended for approximately one minute using a suitable blender to produce a uniform slurry. Let the sample stand at room temperature for 10 - 15 minutes to enhance solvent contact with tissue.

   d. Add 20 ± 1 mL of methanol to a clean 50 mL polypropylene centrifuge tube. Rinse the probe by immersing it in this solution and blending for at least 30 seconds. Use this solution as the second 20 mL rinse in Step F.2.g. The probe must be cleaned between samples with methanol and water rinses, and given a final methanol rinse. A detergent may also be used to assist in cleaning mechanical homogenizers (e.g. UltraTurrix/Polytron).

e. Centrifuge the tissue slurry at approximately 1500 RCF for 10 minutes. Exact speed and centrifugal force is not critical provided a good sediment pack is obtained. Refrigeration may be used, but is not necessary.

f. Decant the supernatant into a 100 mL graduated mixing cylinder or other appropriate graduated glassware.

g. Add the 20 mL of methanol from Step F.2.d to the tissue, vigorously suspend the centrifuged pack with a spatula, centrifuge as in step e above, and add the second supernatant to the first. Combined extracts will be cloudy.

h. Repeat step g, using a fresh 20 mL portion of methanol rather than a rinse, adding the third supernatant to the first and second.

i. Dilute the combined supernatants to 60 ± 1 mL with methanol and mix well.

Note: This is a suitable stopping point. Extracts may be stored for 7 days at 2 - 8 °C.

j. Pipette 8.0 ± 0.1 mL of the combined supernatant into a 15 mL polypropylene tube. Evaporate the sample to less than 0.5 mL under air or nitrogen at 49 ± 2 °C.

Note: Dry the sample aliquot until less than 0.5 mL remains. This is sufficient to remove most of the methanol. A thin film of oily residue will remain on the side of the test tube.

k. Add 2 mL of borate buffer and mix briefly. A dispenser or disposable pipette is sufficiently accurate to use for the buffer addition.

3. Liquid/Liquid Extraction

a. Add 7 mL of ethyl acetate to the test tube containing the sample and vortex for at least 30 seconds. A dispenser or disposable pipette is sufficiently accurate to use for the ethyl acetate addition.

b. Centrifuge the tube for 5 minutes at approximately 800 RCF.

c. Transfer the upper layer (ethyl acetate) into a clean 16 x 100 mm test tube using a disposable pipette or other suitable means, taking care to remove as much of the layer as possible *without removing any of the lower fraction* (borate buffer).

d. Repeat steps 3.a. and 3.b. with fresh ethyl acetate. To save time, do not transfer the upper layer. Proceed to solid phase extraction.

4. Solid Phase Extraction

Note: Steps a. - b. should be performed continuously without letting the cartridges run dry.

a. Wet an acidic alumina SPE cartridge using approximately 5 mL of ethyl acetate. Let the solvent drain to the surface of the cartridge. Flow rate is not important.

b. Transfer the second ethyl acetate fraction from step 3.d. to the cartridge; follow with the first ethyl acetate fraction from step 3.c. Drain the combined ethyl acetate fractions to the surface of the SPE cartridge at a flow rate of approximately 2-4 mL/minute using vacuum if necessary.

c. For muscle only.

i. Wash the cartridge with approximately 5 mL of ethyl acetate at approximately the same flow rate as the sample application and stop the flow when the liquid reaches the surface of the cartridge packing.

ii. Discard the cartridge effluent.

d. To elute ractopamine from the cartridge, add approximately 10 mL of methanol to the cartridge and collect the effluent in a 15 mL polypropylene tube or equivalent vessel. Force the liquid completely from the cartridge using either pressure or vacuum if necessary. Flow rate of methanol should be no greater than approximately 5 mL/minute.

Note: This is a suitable stopping point. The methanol effluent may be stored for 14 days at 2 - 8 °C if needed, before continuing with the method.

e. Begin evaporating the sample using an air or nitrogen stream and a water bath or heater set at 49 ± 2 °C. When 2 mL remain in the tube, remove from the evaporator and vortex for 30 seconds at sufficient speed so that the solvent rises above the 10 mL mark of the centrifuge tube. Return the tube to the evaporator and finish evaporating the solution to dryness.

f. For Liver

Dissolve the sample in 0.5 mL of methanol by swirling the tube or vortexing vigorously for 15 - 30 seconds, and sonicating for approximately 15 seconds in an ultrasonic water bath. Add 0.5 mL sample diluent and mix.

g. For muscle

Dissolve the sample in 1.0 mL of sample diluent by swirling the tube or vortexing vigorously for 15 - 30 seconds, and sonicating for approximately 15 seconds in an ultrasonic water bath.

h. Filter the sample through a PVDF 0.22 µm, 13 mm syringe filter using a small disposable syringe and collect the filtered sample in a HPLC autosampler vial.

Note: This is a suitable stopping point. Samples may be stored for 4 days at 2 - 8 °C if needed, until the sample analysis is completed.

5. Instrumental Settings

Note: The instrument parameters may be optimized to ensure system suitability.

a. HPLC settings

i.	Column:	4.6 mm i.d. x 25 cm Supelcosil LC-18-DB, (guard column may be used)
ii.	Excitation:	226 nm
iii.	Emission:	305 nm
iv.	Flow Rate:	1.0 mL/minute
v.	Injection Volume:	100 µL
vi.	Column Temperature:	Ambient (20 - 25 °C)
vii.	Run Time:	10 minutes
viii.	Mobile Phase:	Refer to section C.2.c.

b. Initial test for HPLC system suitability (to be used when initiating analysis for the first time, or whenever instrumentation, HPLC column, or elution parameters are changed, or degradation of instrument performance is suspected.

i. Inject an appropriate resolution solution and external standard curve solutions for liver and muscle analysis, and verify system meets requirements specified in items ii – vi below.

ii. Ractopamine must be baseline resolved from ritodrine. See Figure 1, Section J.Appendix 2, for a sample chromatogram.

iii. The signal/noise ratio of the 25 ng/mL standard for a liver set, or the 0.5 ng/mL standard for a muscle set should be at least 5.

iv. The ractopamine peak should be well resolved from the solvent front and elute between 4 - 8 minutes.

v. The ractopamine retention time range for all injected standards (minimum to maximum) should not exceed 3% of the average value.

vi. The correlation coefficient that is calculated for the standard curve(s) must be ≥0.995.

Note: Decreasing acetonitrile concentration in the mobile phase increases the resolution of ractopamine from ritodrine and may separate impurities co-elution with ractopamine. Mobile phase composition should be optimized for each HPLC/column system.

c. Daily HPLC suitability test (to be run before analyzing samples)

Check system suitability by injecting standard curve(s) appropriate for the day's analysis. Verify that system meets requirements specified above in section b. iii – vi.

6. Injection sequence (if applicable)/Sample Set
   a. Resolution standard (optional)
   b. Standards
   c. Recovery
   d. Blank
   e. Samples, up to a maximum of 24
   f. At least one standard

   Note: It is recommended that the column be flushed with a strong solvent/water mixture (acetonitrile or MeOH/water, 80:20) after the end of the analytical batch to remove residual matrix from the column.

G. CALCULATIONS / IDENTIFICATION

1. Using linear regression analysis, calculate the slope, intercept, and correlation coefficient of a standard curve constructed by plotting peak areas versus concentration (ng/mL) for all of the injected standards.

   Note: Standard curve must have a correlation coefficient (r-value) ≥ 0.995 over the concentration range used for quantification.

2. The concentration of ractopamine HCl can be calculated using the following equation:

   $$\text{ppb Ractopamine HCl} = \frac{[(A-B)xE]}{(CxF)}$$

   A = HPLC peak area of sample injection

   B = Intercept from the calibration curve

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

   E = Total volume (mL) = (Initial volume/aliquot volume) x final volume

   F = Weight of tissue sample (g)

   Note: Area response should be linear with respect to the concentration of ractopamine HCl. (Refer to section J.5. in the event acceptable recovery and blank control results are not obtained).

3. Results may be reported only if the following conditions have been met:
   a. The correlation coefficient calculated for the standard curve is ≥ 0.995
   b. No quantifiable peak eluting within ractopamine's elution time window is detected

in the blank sample

c. The recovery of the positive control falls within the limits specified in section I.1.

d. Measure the HPLC retention times and peak areas for ractopamine in all sample set chromatograms.

e. Identify a peak in any sample as ractopamine if its retention time matches that of an appropriate reference standard within ± 3.0%.

f. If the peak area for ractopamine in a sample exceeds the high end of the standard curve, the final extract should be diluted and re-injected along with one standard curve set. Another option is to take a smaller aliquot of the initial methanol extract and reprocess the sample. If the amount found exceeds known SPE cartridge capacity, the sample should be reprocessed using a smaller aliquot (step F.2.j) in order to assure a consistent recovery.

Note: The capacity of Waters Sep-Pak Plus Alumina A cartridges has been verified with 150 ppb recovery samples using 4x the recommended aliquot of the combined supernatant (step F.2.j).

g. Verify that the standard injected at the end of the set meets the system suitability criterion listed in section F.5.b.iii.

H. SAFETY INFORMATION AND PRECAUTIONS

1. Required Protective Equipment — Safety eyewear, protective gloves, and lab coat.

2. Hazards

Procedure Step	*Hazard*	*Recommended Safe Procedures*
Glacial acetic acid	Strong acid	Wear protective equipment, avoid contact with skin.
Ractopamine HCl	Eye irritant and exposure may increase heart rate.	Wear protective equipment, avoid breathing powder.
Ritodrine HCl	Irritant, fast or irregular heartbeat, nausea, shortness of breath.	See ractopamine HCl above.
Methanol, Ethyl Acetate	Flammable	Keep in well-closed containers in a cool place and away from fire. Use it in well-ventilated hood.

3. Disposal Procedures

Follow local, state and federal guidelines for disposal.

I. QUALITY ASSURANCE PLAN

1. Performance Standard

Analyte	*Tissue*	*Analytical Range*	*Acceptable Recovery*	*Acceptable Repeatability (CV)*
Ractopamine HCl	Liver	25 - 300 ppb	60 - 115 %	≤ 20 %
Ractopamine HCl	Muscle	1.0 - 300 ppb	60 - 115 %	≤ 20 %

Acceptable correlation coefficient for standard curve: ≥0.995.

2. Critical Control Points and Specifications

Record	*Acceptable Control*
Sample weight	10.0 ± 0.2 g
Methanol	20 ± 1 mL
Combined methanol supernatant	60 ± 1 mL
Aliquot volume	8 ± 0.1mL
Water bath temperature	49 ± 2 °C

3. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: One per week per analyst when samples analyzed.

ii. Records are to be maintained.

b. Acceptability criteria.

Refer to I. 1.

If unacceptable values are obtained, then:

i. Investigate following established procedures.

ii. Take corrective action as warranted.

4. Sample Condition upon Receipt

   Cold or frozen

J. APPENDIX

1. References

   Animal Drug Application (NADA), Laboratory Procedure for Method B03858 Revision 02, Determination of Ractopamine Hydrochloride in Cattle Tissues by High Performance Liquid Chromatography. Effective 12/14/1998.

2. Chromatograms/spectra

   Figure 1. Resolution of ractopamine and ritodrine (2.5 ng each injected on column) using a Supelcosil LC-18-DB column.

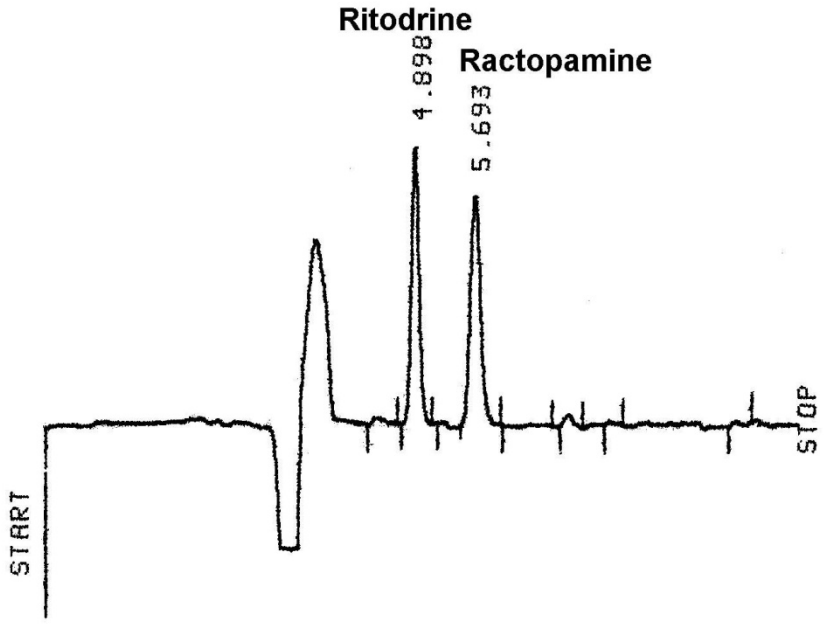
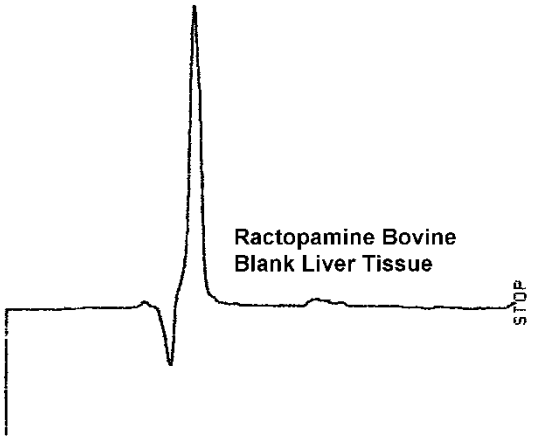


Figure 2. Chromatograms showing blank and fortified liver tissues, respectively



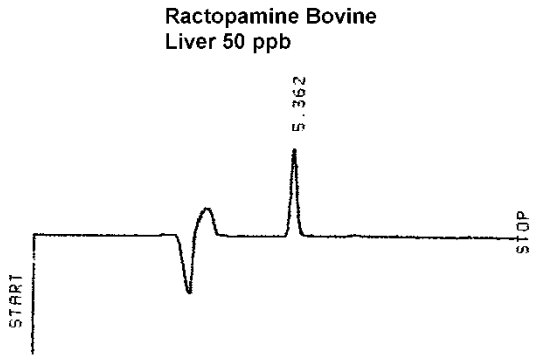
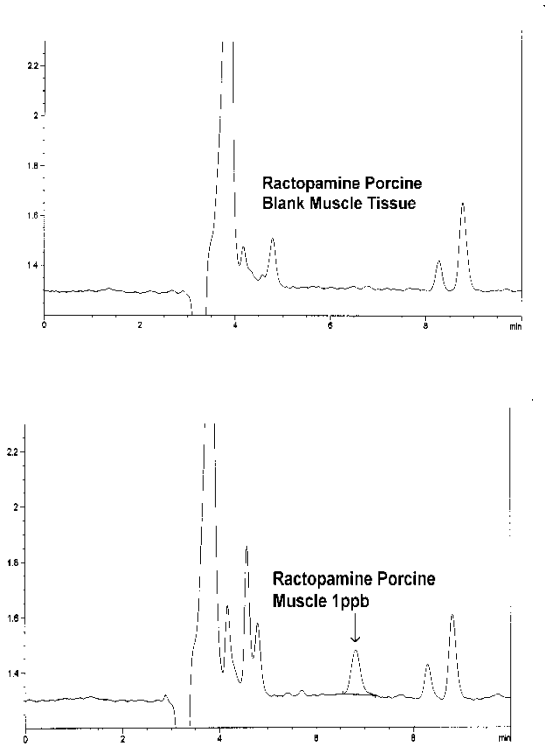
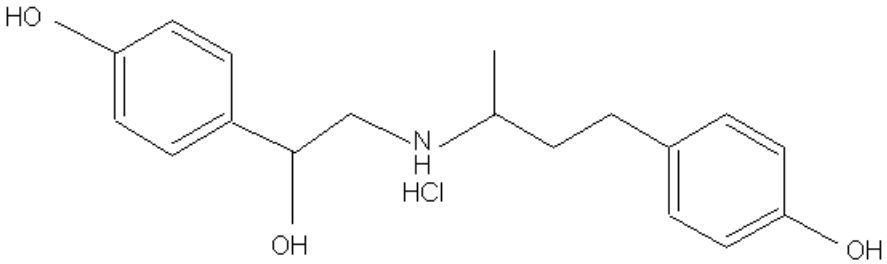


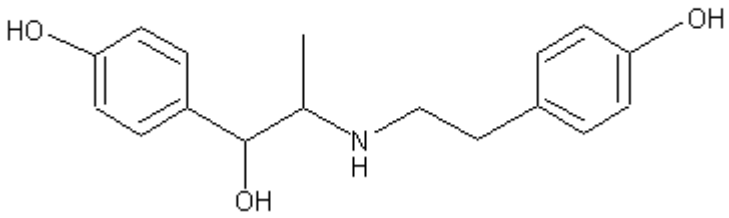
Figure 3. Chromatograms showing blank and fortified 1 ppb muscle tissues, respectively



3. Structure of ractopamine HCl and ritodrine.



Structure of Ritodrine



4. Alternative HPLC column - C18 reversed phase HPLC column Beckman Ultrasphere IP, 5 µm, 4.6 x 250 mm, Part No. 235335.

5. SPE cartridge testing

In the event that acceptable standard recovery (> 60 %) and control results are not attainable using the method; the following steps may be taken to determine the suitability of the acidic alumina SPE cartridges. As a preventative measure, each new lot number of SPE cartridges may be tested using test procedure 1 to determine their suitability.

Note: 2 g acidic alumina activity I from Waters, Alltech Isolute, and ICN proved to be acceptable using this test.

a. Preparation of Test Solutions

i. Ractopamine HCl fortification solution (0.2 µg/mL):

Pipet 2 mL of the 10 µg/mL ractopamine HCl intermediate muscle solution into a 100 mL volumetric flask and dilute to volume with sample diluent. Mix well. This solution is stable for one month when stored at 2 - 8 °C.

ii. Ractopamine HCl external standard (2.5 ng/mL):
Pipet 10 mL of the 25 ng/mL ractopamine HCl muscle standard, or 2.5 mL

of the 100 ng/mL ractopamine HCl intermediate muscle standard, into a 100 mL volumetric flask and dilute to volume with sample diluent. This solution is stable for one month when stored at 2 - 8 °C.

b. Test Procedure 1

i. Fortify 2 mL of Borate Buffer with 0.1 mL of the 0.2 µg/mL fortification standard (20 ng).

ii. Perform steps F.3.a through F.4.f and analyze as described in the method.

iii. At least 85% (17 ng) of the fortification should be recovered.

iv. If 85% is not recovered, a new source of acidic alumina should be tested.

c. Test Procedure 2.

The suitability may further be evaluated using the following procedure, which will determine the SPE performance in the presence of control and fortified liver tissue extracts.

i. Prepare control extracts of tissue following steps F.2.a through k.

ii. Fortify the desired number of samples with 0.1 mL of a 0.2 µg/mL ractopamine HCl fortification standard (20 ng).

Note: Addition of 20 ng ractopamine HCl at the borate buffer stage is equivalent to 15 ppb in tissue.

iii. Perform steps F.3.a through F.4.f and analyze as described in the method.

iv. At least 80% (16 ng) of the fortified analyte should be recovered.

v. The area of the tissue blank at the retention time of ractopamine should be less than 20% of the area obtained when injecting 100 µl of the 2.5 ng/mL external standard.

vi. If 80% is not recovered, a new source of acidic alumina should be tested.

K. APPROVALS AND AUTHORITIES

1. Approvals on file.

2. Issuing Authority: Director, Laboratory Quality Assurance Division.