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Title: Screening, Determination, and Confirmation of Beta-Agonists by HPLC/MS/MS		
Revision: 08	Replaces: CLG- AGON1.07	Effective: 01/20/2016

Contents

A. INTRODUCTION

1. Background

Beta-agonists are also known as beta-adrenergic agonists. An adrenergic agent is a drug that has effects similar to epinephrine (adrenaline). Adrenergic drugs either stimulate a response (agonists) or inhibit a response (antagonists). The five categories of adrenergic receptors are: α1, α2, β1, β2, and β3, and agonists vary in specificity between these receptors, and may be classified respectively. Thus, beta-agonists stimulate a response of the beta receptors.

Beta-agonists are used for growth promotion in food animals, increasing lean muscle mass. In humans, clenbuterol and salbutamol are used as bronchodilators by asthma sufferers and as performance-enhancing drugs by athletes. Human side effects include increased heart rate and blood pressure, anxiety, palpitation, and skeletal muscle tremors.

2. Summary of Procedure

Free residues of clenbuterol, salbutamol, cimaterol, zilpaterol, and ractopamine are extracted from liver or muscle tissues with a mixture of acetonitrile and isopropanol. Sodium chloride, sodium sulfate, and magnesium sulfate salts are used to precipitate proteins and dehydrate the solution. This extract is evaporated, reconstituted in water, filtered, and analyzed by HPLC/MS/MS.

3. Applicability

This method is suitable for the screening, confirmation and/or quantitation of β-agonists at the levels listed in Tables 1 and 2.

Table 1 –Screening and Confirmation levels

	Bovine Muscle (ppb)	Porcine Muscle (ppb)	Equine Muscle (ppb)	Bovine Liver (ppb)	Porcine Liver (ppb)
clenbuterol	≥ 3	≥ 3	≥ 3	≥ 3	≥ 3
salbutamol	≥ 3	≥ 3	≥ 3	≥ 3	≥ 3
cimaterol	≥ 3	≥ 3	≥ 3	≥ 3	≥ 3
zilpaterol	≥ 6	≥ 6	≥ 6	≥ 6	≥ 6
ractopamine ● HCl	≥ 15	≥ 15	≥ 15	≥ 15	≥ 15

Table 2 –Quantitation levels

	Bovine Muscle (ppb)	Porcine Muscle (ppb)	Equine Muscle (ppb)	Bovine Liver (ppb)	Porcine Liver (ppb)
zilpaterol	≥ 25	N/App	N/App	≥ 75	N/App
ractopamine ● HCl	≥ 15	≥ 15	N/App	≥ 45	≥ 45

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. Petri Dishes – disposable polystyrene, Optilux 100 x 20 mm, Cat. No. 1005, Becton Dickinson.
   b. Waring commercial blender Model 51BL31.
   c. Tissuemizer – Polytron, Kinematica AG, Model No. PT2100.
   d. Top loading balance – 0.01 g sensitivity, PJ3600 DeltaRange, Mettler.
   e. Centrifuge tubes – 50 mL round bottom polyallomer with sealing caps, Cat. No. 3138-0050, Nalgene.
   f. Centrifuge tubes – 50 mL conical, disposable, polypropylene, with caps, Cat. No. 352098, Becton Dickinson.
   g. Micropipettes – Adjustable, 10 – 5000 µL, Eppendorf.
   h. Glass rods – 10 mm diameter by 25 cm length, fired and rounded at both ends.
   i. Vortex mixer – variable speed, Cat. No. S8223-1, American Scientific Products.
   j. Shaker – Horizontal flatbed, two speed, Cat. No. 511105, Eberbach.
   k. Centrifuge – International Equipment Company B-22M high speed with rotor 876 for 50 mL tubes, Cat. No. 20671-007, VWR Scientific.
   l. Syringeless filter device – Mini-UniPrep, 0.2µm nylon, Cat. No. UN203NPUNYL, Whatman.
   m. Evaporator – N-Evap 112, Organomation Associates Inc., Model No. 8125.
   n. Filter – 0.2 µm, nylon, acrodisc-13, Cat. No. 4551, Pall Gelman Sciences, Inc.
   o. Amber autosampler vial, 12 x 32 mm, E&K, Cat. No. E251011
   p. Analytical balance – 0.0001 g sensitivity, AG204, Mettler.

q. Volumetric flasks –100 mL amber, 10 mL amber.

r. Graduated cylinder– 1L, Class A.

s. Food processor – Robot Coupe model RSI6Y-1, Robot Coupe USA Inc.

t. Sample cups – eValue 4.5 oz specimen containers with caps, Cat. No. C686550, E&K Scientific.

2. Instrumentation

a. Varian 212-LC equipped with Varian 460-LC autosampler and Varian Prostar 500 Column heater.

b. Varian 325 mass spectrometer.

c. HPLC guard column - C18 Security Guard cartridge, 2.1 x 4 mm, 2 µm particles, Cat. No. AJO-4286 and Security Guard kit, Cat. No. KJO-4282, Phenomenex.

d. HPLC column – Kinetex C18, 100 x 2.1 mm, 2.6 µm particles, Cat. 621769-48, Phenomenex. (for Salbutamol, Cimaterol, Clenbuterol, and Ractopamine).

e. HPLC column - BetaSil CN, 2.1 x 100 mm, 5 µm particles, Cat. No. 70805-102130, Thermo Scientific (for Zilpaterol).

f. HPLC Column- DyChrom Chemosorb 5CN-U(R) 2.1 mm x 100 mm, Catalog No. G2R03. DyChrom.

C. REAGENTS AND SOLUTIONS

Note: Equivalent reagents / solutions may be substituted. The stability time frame of the solution is dependent on the expiration dates of the compounds used. The maximum length of time that a working reagent shall be used is 1 year unless the laboratory has produced extension data.

1. Reagents

a. Methanol (MeOH) – HPLC grade, Cat. No. 230-4, Burdick & Jackson

b. Acetonitrile (ACN) – HPLC grade, Cat. No. 015-4, Burdick & Jackson.

c. Isopropanol (IPA) – HPLC grade, Cat. No. AH323-4, Burdick & Jackson.

d. Sodium chloride (NaCl) – ACS reagent grade, Cat. No. S271-1, Fisher.

e. Sodium sulfate (Na_2SO_4) – ACS reagent grade, Cat. No. 354250010, Acros Organics.

f. Magnesium sulfate ($MgSO_4$) – anhydrous, minimum 99.5% purity, Cat. No. M-7506, Sigma.

g. Pre-weighed salts (NaCl, $MgSO_4$, Na_2SO_4) – Cat. No. ECQUUS8-MP2, UCT.

h. Water – Deionized, HPLC grade, Millipore Rx system.

i. Formic acid – Purity 98 - 100%, Cat. No. 27001, Riedel-de Haën.

2. Solutions

   a. Aqueous mobile phase (0.1% formic acid in water):

      Pipette 1 mL of formic acid into a 1L class A graduated cylinder. Fill to volume with Millipore water.

   b. 4:1 Acetonitrile/Isopropanol Solution

      Add 800 mL Acetonitrile and 200 mL Isopropanol into a 1L class A graduated cylinder.

D. STANDARD(S)

Note: Equivalent standards / solutions may be substituted. Purity and counterions are to be taken into account when calculating standard concentrations. The stability time frame of the solution is dependent on the expiration date of the components used. In-house prepared standards shall be assigned an expiration date that is no later than the expiration date of the earliest expiring component or no later than the stability stated in the method, whichever ends soonest. The maximum length of time that an in-house prepared standard shall be used is 1 year unless the laboratory has produced extension data.

1. Standard Information

   a. Clenbuterol (CLEN) ● HCl– approximately 95% pure, Cat. No. C-5423, Sigma Chemical Co.

   b. Cimaterol (CIM) – Cat. No. 159757, MP Biochemicals Inc.

   c. Salbutamol (SAL) – approximately 95% pure, Cat. No. S-8260, Sigma Chemical Co.

   d. Ractopamine ● HCl (RAC) – Elanco Animal Health.

   e. Ractopamine-d6-HCl – Cat. No. R071402, TRC Inc.

   f. Zilpaterol (ZIL) – Intervet Inc.

   g. Zilpaterol- d7– Cat. No. Z430002, TRC Inc.

2. Preparation of Standard Solution(s)

   Note: Adjust all standard weights for purity. The concentration of Clenbuterol ● HCl is to be corrected for salt, since Clenbuterol is the analyte of interest as opposed to Clenbuterol ● HCl. The Ractopamine ● HCl concentration is not to be corrected for salt since Ractopamine ● HCl is the analyte of interest. See calculation in section G.

   a. CLEN, CIM, SAL and ZIL stock standards (~25 µg/mL):

      Weigh ~2.5 mg of CLEN, CIM, SAL and ZIL into its own 100 mL amber volumetric flask and bring to volume with methanol. Record the weight to 0.1 mg and calculate the exact concentration. These standards are stable for 2 months when stored in a refrigerator at 2 - 8 °C.

b. RAC stock standard (~1 mg/mL):

Weigh ~10.0 mg into a 10 mL volumetric flask and dilute to volume with methanol. Record the weight to 0.1 mg and calculate the exact concentration. This standard is stable for 3 months when stored in a refrigerator at 2 - 8 °C.

c. RAC intermediate standard (10 µg/mL):

Pipette ~100 µL (adjusted for the actual stock standard concentration) of the RAC stock standard into a 10 mL volumetric flask and bring to volume with Millipore water. This standard is stable for 1 month when stored in a refrigerator at 2 - 8 °C.

d. Mixed intermediate standard A (250 ng/mL CLEN, CIM, SAL, 1250 ng/mL RAC; 500 ng/mL ZIL):

Pipette ~100 µL (adjusted for the actual stock standard concentration) of the CLEN, CIM, SAL stock standards, ~200 µL of the ZIL stock standard, and ~1250 µL of the 10 µg/mL RAC intermediate standard into a 10 mL amber volumetric flask and bring to volume with Millipore water. This standard is stable for 1 month when stored in a refrigerator at 2 - 8 °C.

e. Mixed intermediate standard B (50 ng/mL CLEN, CIM, SAL, 250 ng/mL RAC; 100 ng/mL ZIL):

Pipette 200 µL of the mixed intermediate standard A into an HPLC vial and add 800 µL Millipore water. This standard is stable for 1 month when stored in a refrigerator at 2 - 8 °C.

f. Mixed external standard (3 ng/mL CLEN, CIM, SAL, 15 ng/ mL RAC; 6 ng/mL ZIL):

Pipette 60 µL of mixed intermediate standard B and 940 µL of Millipore water into an HPLC vial. This standard is stable for 1 month when stored in a refrigerator at 2 - 8 °C.

g. Quantitation spiking standard for muscle (1.5 µg/mL RAC and 0.5 µg/mL ZIL):

Pipette 1.5 mL of RAC intermediate standard (adjusted for the actual stock standard concentration)and 0.20mL of ZIL stock standard (adjusted for the actual stock standard concentration)into a 10 mL amber volumetric flask and bring to volume with a 4:1 acetonitrile / Isopropanol solution. This standard is stable for 1 month when stored in a refrigerator at 2 – 8 °C. For calibration curve preparation, refer to table 3 in section F.2 for detail.

h. Quantitation spiking standard for liver (3.75 RAC µg/mL and 0.5 µg/mL ZIL):

Pipette 3.75 mL of RAC intermediate standard (adjusted for the actual stock standard concentration) and 0.20 mL of ZIL stock standard (adjusted for the actual stock standard concentration into a 10 mL amber volumetric flask and bring to volume with a 4:1 acetonitrile/isopropanol solution. This standard is stable for 1 month when stored in a refrigerator at 2-8°C. For calibration curve

preparation, refer to table 3 in section F.2 for details

i. ZIL-D7 stock standard (~20 µg/mL) :

Weigh ~1 mg of ZIL into a 50 mL amber volumetric flask and bring to volume with a 4:1 Acetonitrile / Isopropanol Solution. Record the weight to 0.1 mg and calculate the exact concentration. These standards are stable for 1 months when stored in a refrigerator at ≤ -20 °C.

j. RAC-D6 stock standard (100 µg/mL):

Weigh 1 mg of RAC-D6 into a 10 mL volumetric flask and bring to volume with a 4:1 Acetonitrile / Isopropanol Solution. This standard is stable for 1 year when stored in a refrigerator ≤ -20°C.

k. RAC-D6 (5 µg/mL) and ZIL-D7 (2 µg/mL) Internal standard:

Pipette 0.5 mL of ZIL-D7 stock standard and 0.25 mL of RAC-D6 into a 5 mL amber volumetric flask and bring to volume with volume with a 4:1 acetonitrile/isopropanol solution. This standard is stable for 1 month when stored in a refrigerator at 2-8 °C.

E. SAMPLE PREPARATION

1. Liver and Muscle homogenization

   a. Blender or food processor

      i. Cut liver or muscle sample into smaller pieces and homogenize in a blender or food processor.

      ii. Transfer homogenized sample into a plastic bag and store in a freezer at ≤ -10 ºC.

      iii. Let sample partially thaw prior to analysis.

   b. Alternatively, dry ice grinding can be used for muscle sample homogenization

      i. Chop 0.5 - 1 lb of muscle tissue into small pieces and homogenize with an equal amount of dry ice in a large food processor. The resulting sample homogenate will be a frozen powder.

      ii. Transfer a portion of the homogenized sample into a loosely capped sample cup until the dry ice has sublimed. Excess sample from step b.i.may be discarded.

      iii. Tighten the caps and store in a freezer at ≤ -10 ºC .

F. ANALYTICAL PROCEDURE

1. Preparation of Controls for Liver and Muscle Tissue for Screening and Confirmation.

a. Weigh two 5.0 ± 0.1 g blank homogenized tissue portions into 50 mL disposable centrifuge tubes.

b. Prepare positive and negative controls by fortifying one with 60 µL of the mixed intermediate standard A (D.2.d.).The remaining portion is fortified with internal standard as a negative control.

2. Preparation of Calibration curve and Controls for Liver and Muscle Tissue for Quantitation.

a. Weight six - 5.0 ± 0.1 g blank homogenized liver or muscle tissue portions into 50 mL disposable centrifuge tubes. More may be required for additional QC with large batches.

b. Prepare a calibration curve for the compound of interest with at least 4 levels that bracket the targeted level. See Table 3.

c. Prepare a positive control by fortifying one portion at 0.5X. See Table 3.

d. The remaining portion is fortified with internal standard as a negative control.

e. Bring the total volume to 5 mL with a 4:1 Acetonitrile / Isopropanol solution. See Table 4.

Table 3 – Calibration Curve

		Ractopamine				Zilpaterol		Zilpaterol-D7 (IS)	Ractopamine -D6 (IS)
Spiking levels		Bovine		Porcine		Bovine			
		Muscle	Liver	Muscle	Liver	Muscle	Liver		
0.2X	Volume (µL)	20.0	24.0	33.3	40.0	20.0	24.0	10	25
0.5X	Volume (µL)	50.0	60.0	83.3	100.0	50.0	60.0	10	25
X	Volume (µL)	100.0	120.0	166.7	200.0	100.0	120.0	10	25
1.5X	Volume (µL)	150.0	180.0	250.0	300.0	150.0	180.0	10	25
2X	Volume (µL)	200.0	240.0	333.3	400.0	200.0	240	10	25

Table 4 –Additional dilution volume for quantitation standards

		Ractopamine				Zilpaterol	
Spiking levels		**Bovine**		**Porcine**		**Bovine**	
	4:1 ACN/IPA	**Muscle**	**Liver**	**Muscle**	**Liver**	**Muscle**	**Liver**
0.2X	Volume (mL)	4.98	4.98	4.97	4.96	4.98	4.98
0.5X	Volume (mL)	4.95	4.94	4.92	4.90	4.95	4.94
X	Volume (mL)	4.90	4.88	4.83	4.80	4.90	4.88
1.5X	Volume (mL)	4.85	4.82	4.75	4.70	4.85	4.82
2X	Volume (mL)	4.80	4.76	4.67	4.60	4.80	4.76

3. Sample Extraction for Liver and Muscle Tissue

   a. Weigh 5.0 ± 0.1 g homogenized tissue into a 50 mL disposable centrifuge tube. Include the prepared positive and negative controls in the sample set at this time. **For quantitation only: spike the samples with 10 µL of Zilpaterol-D7 and 25 µL of Ractopamine-D6 Internal Standard.**

   b. Add 4 mL acetonitrile and 1 mL isopropanol. For dry ice ground muscle (E.1.b.), cap and shake or vortex for 2 minutes. For muscle homogenized without dry ice (E.1.a), tissuemize for 30 seconds.

   c. Add 1.2 g NaCl and shake or vortex for 2 minutes.

   d. Add 4 g Na_2SO_4 and 0.5 g $MgSO_4$ and shake or vortex for 2 minutes.

   Note: This is a suitable stopping point. Samples may be stored overnight at 2 - 8 °C.

   e. Centrifuge the samples for about five minutes at approximately 2000 RCF.

   f. Filter extract by either of:

      i. Whatman mini-uniprep filter vial.

         (a) Pipette 0.5 mL of extract into Whatman mini-uniprep filter vial.

         (b) Evaporate to dryness with air or nitrogen.

         (c) Add 0.5 mL Millipore water to vial and filter reconstituted extract by pushing plunger-shaped cap equipped with a 0.2µm nylon filter into vial.

      ii. 3 mL syringe and 0.2µm nylon filter.

         (a) Pipette 0.8 mL of extract into a 12x75 mm glass tube.

         (b) Evaporate to dryness with air or nitrogen.

(c) Add 0.8 mL of Milli-Q water to the glass tube, vortex for 30 seconds and filter using a 3 mL syringe and 0.2µm nylon filter into a vial.

4. Instrumental Settings

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

a. HPLC Conditions:

Aqueous Mobile Phase	0.1% formic acid in water
Organic Mobile Phase	Acetonitrile
Flow Rate	0.3 mL/min
Column Temperature	25 °C
Injection Volume	50 µL
Run Time	12 minutes

b. HPLC Mobile Phase Gradient Table:

Time	% Aqueous	% Organic
0:00	95%	5%
1:30	95%	5%
7:30	35%	65%
7:36	95%	5%
12:00	95%	5%

c. Interface Conditions:

Varian

Ion Mode	ES+
Needle Voltage	400 °C
Drying gas	300 °C
Vortex gas	150 °C
Nebulizer Pressure	55 psi
Drying gas Pressure	20 psi
Vortex pressure	10 psi

d. MRM Parameters:

	Precursor Ion (m/z)	Product Ion (m/z)*	Collision Energy (eV)
Salbutamol	240	**148**	18
		166	14
		121	26
Cimaterol	220	116	33
		143	23
		160	16
Clenbuterol	277	168	32
		203	15
		132	30
Ractopamine	302	107	27
		121	21
		164	16
Zilpaterol	262	157	33
		185	25
		130	50
Ractopamine -D6	308	290	12
		121	20
		168	16
Zilpaterol-D7	269	**251**	13
		185	25
		203	19

* Most abundant product ion (quant ion) is in bold.

e. MS Parameters:

Segment #	1	2
Starting Retention Time (min)	0.0	4.0
Dwell Time (sec)	0.2	0.2
Capillary (kV)	1	1
Multiplier (V)	650	650
Analytes	SAL, CIM, & ZIL	CLEN & RAC

5. Injection sequence/Sample Set
   a. External Standard or Calibration Curve
   b. Matrix Match Sample (Quantitation)
   c. Positive Control
   d. Water Blank
   e. Negative Control
   f. Intra-laboratory check sample (if needed)
   g. Samples, up to a maximum of 48

The external standard, positive control and negative control must be re-injected after a maximum of 24 samples. The positive control shall also be re-injected at the end of the injection sequence.

G. CALCULATIONS / IDENTIFICATION

1. Calculations

   a. Correction of Clenbuterol Concentration for Salt and Purity

$$\text{Mass}_{\text{Clenbuterol}} = \text{Mass}_{\text{Clenbuterol} \bullet \text{HCl}} \times \frac{\text{Molecular Mass}_{\text{Clenbuterol}}}{\text{Molecular Mass}_{\text{Clenbuterol} \bullet \text{HCl}}} \times \text{Purity}$$

   Where $\text{Mass}_{\text{Clenbuterol} \bullet \text{HCl}}$ = ~2.5 mg

   $\text{Molecular Mass}_{\text{Clenbuterol}}$ = 277.19 g/mol

   $\text{Molecular Mass}_{\text{Clenbuterol} \bullet \text{HCl}}$ = 313.65 g/mol

   Purity = 0.95 (for the product listed)

2. Estimated Amount Found

This is a quantitative estimate calculated for comparison to the screen cutoff level. It is based on a one point calibration with the positive control injected most recently before the sample as the reference. The MS instruments can be programmed to automatically do this calculation.

D = E * B sample / B pos. ctrl.

Where D = Estimated Amount Found in the Sample (ppb)

E = Positive Control Fortification Level (ppb)

B sample = Quant Ion Peak Area in the Sample (counts)

B pos. ctrl. = Quant Ion Peak Area in the Positive Control injected most recently before the sample (counts)

3. Screen Cutoff Level

This level is used to determine if a sample is screen positive or negative. It is calculated using the formulas below:

F = E * H

Where F = Screen Cutoff Level (ppb)

E = MLA (ppb)

H = Minimum / Maximum Recovery (unitless)

Take values from Table 1 below.

Note: Values in Table 5 may be updated as more data becomes available.

This is a safety factor to ensure that violations are not missed due to variation in recovery. These values are based on the positive control having the maximum recovery and the sample having the minimum recovery.

Table 5 – Minimum / Maximum Recovery Values

Cmpd #	Name	Min / Max recovery
1	Clenbuterol	0.40
2	Cimaterol	0.62
3	Salbutamol	0.67
4	Ractopamine ● HCl	0.47
5	Zilpaterol	0.73

4. Quantitation Calculation

   a. Peak areas of analytes and internal standards are used for quantitation.

   b. Calculate the regression parameters for the calibration curve using the linear regression formula,

      $y = mx + b$

      where:

      y= area ratio of the analyte/internal standard

      x= concentration of standards (ppb)

   c. The coefficient of correlation (r^2) must be ≥ 0.99.

   d. Do not use the origin as a data regression point.

   e. Determine sample concentrations using the linear regression formula.

5. Screening Criteria

   a. The retention time of each analyte must match that of the positive control or the external standard injected most recently before the sample within 5%.

   b. All product ions for a given analyte must be present. The required ions are listed in F.5.d.

   c. Each ion must have a signal-to-noise ratio ≥ 3.

   d. The sample is screen positive if the estimated amount found equals or exceeds the screen cutoff level (D ≥ F).

   e. A water blank injected immediately after the initial positive control injection must be negative for all analytes according to criteria G.5.a.-c. above.

f. Referring to the negative and positive controls injected most recently before the relevant sample, the negative control must be negative for all analytes according to criteria G.2.a.-c. above, with the quant ion peak area being ≤ 5% of the positive control injection.

g. The positive controls injected closest in time before and after the relevant sample must both be positive for all analytes according to criteria G.5.b.-c. above.

6. Confirmation Criteria

a. The retention time of the analyte(s) must match that of the positive control or the external standard injected most recently before the relevant sample within 5%.

b. Product ion abundance ratios must match that of the positive control or the external standard injected most recently before the relevant sample within a ±20% absolute difference for 3 product ions or ±10% for 2 product ions. The following are representative ion ratios calculated relative to the most abundant product ion:

	Ratio #1	Ratio #2
SAL	166/148	121/148
CIM	116/160	143/160
CLEN	168/203	132/203
RAC	121/164	107/164
ZIL	157/185	130/185

c. Each ion must have a signal-to-noise ratio ≥ 3.

d. A water blank injected immediately after the initial positive control injection must be negative according to screening criteria G.5.a.-c. above.

e. Referring to the negative and the positive controls injected most recently before the relevant sample, the negative control must be negative according to criteria G.2.a.-b. above, with the quant ion peak area being ≤ 5% of the positive control.

f. The positive controls injected closest in time before and after the relevant sample must both be positive according to screening criteria G.5.b.-c. above.

Note: Confirmation criteria for the negative control and positive control are required only for analytes that are to be confirmed in the sample set.

7. Quantitation Criteria

a. The sample peak retention time must be within ± 5% of a standard (1.5x mid-level calibration standard recommended) or positive control.

b. The quantitative ion must have a signal to noise ratio of ≥ 10.

c. The additional ions for the analyte listed in F.4.d. must be present in sample with a signal to noise ratio of ≥ 3.

d. The negative control response must be less than 10% of the positive control run in the same set.

e. The positive control injected after the matrix match sample must be 60-110% for all analytes.

f. The determinative coefficient (r^2) for the calibration curve must be ≥ 0.99.

g. For validation – at a minimum ¾ of the QC samples in analytical run should have recoveries within 60-110%.

Note: Quantitation criteria are required only for analytes that are to be quantitated in the sample set.

H. SAFETY INFORMATION AND PRECAUTIONS

1. Required Protective Equipment — safety glasses and/or face shield, disposable gloves, lab coat.

2. Hazards

Procedure Step	*Hazard*	*Recommended Safe Procedures*
Methanol, acetonitrile, and isopropanol	Flammable and poisonous	Use reagents in an efficient fume hood away from all electrical devices and open flames. Wear gloves and protective eyewear.
Formic acid	Acid burns	Wear protective equipment and avoid contact with skin.

3. Disposal Procedures

Follow local, state and federal guidelines for disposal.

I. QUALITY ASSURANCE PLAN

1. Performance Standard

a. Positive control is positive for all analytes using the criteria in Section G.

b. Negative control is negative for all analytes using the criteria in Section G.

c. For quantitation the positive control recoveries must be 60-110% for all analytes that will be quantitated.

2. Critical Control Points and Specifications

Record	*Acceptable Control*
Sample weight of muscle tissue	5.0 ± 0.1 g

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. Condition upon receipt - Cool or frozen

J. APPENDIX

1. Proposed fragmentation patterns

Clenbuterol

Ion (m/z)	Fragment
277	$[M+H]^+$
203	$[M+H-H_2O-(CH_3)_2C=CH_2]^+$
168	$[M+H-H_2O-(CH_3)_2C=CH_2-Cl]^+$
132	$[M+H-H_2O-(CH_3)_2C=CH_2-Cl-HCl]^+$

Salbutamol

Ion (m/z)	Fragment
240	$[M+H]^+$
166	$[M+H-H_2O-(CH_3)_2C=CH_2]^+$
148	$[M+H-2H_2O-(CH_3)_2C=CH_2]^+$
121	$[M+H-2H_2O-(CH_3)_2C=CH_2-HCN]^+$

Cimaterol

Ion (m/z)	Fragment
220	$[M+H]^+$
160	$[M+H-H_2O-CH_3CH=CH_2]^+$
143	$[M+H-H_2O-(CH_3)_2CHNH_2]^+$
116	$[M+H-H_2O-(CH_3)_2CHNH_2-HCN]^+$

Ractopamine

Ion (m/z)	Fragment
302	$[M+H]^+$
164	$[M+H-CH_2CHOH(C_6H_4)OH]^+$
121	$[M+H-(CH_3)CHNHCH_2CHOH(C_6H_4)OH]^+$
107	$[M+H-CH_2CH(CH_3)NHCH_2CHOH(C_6H_4)OH]^+$

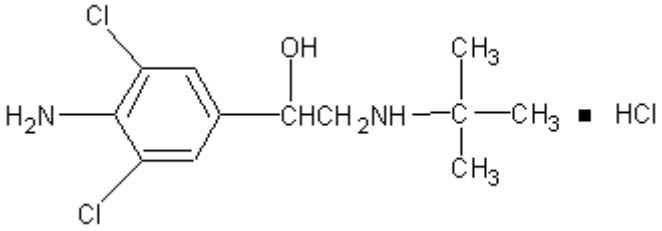
Zilpaterol

Ion (m/z)	Fragment
262	$[M+H]^+$
185	$[M+H-H_2O-(CH_3)_2CHNH_2]^+$
157	$[M+H- H_2O-(CH_3)_2CHNH_2-CO]^+$
130	$[M+H-C_7H_4N_2O$

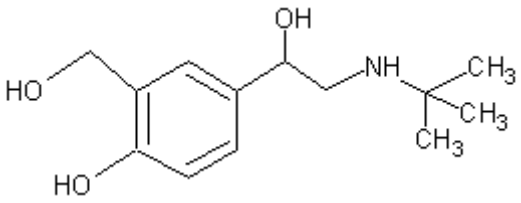
2. Sample Chromatograms and Mass Spectra

{Reserved}

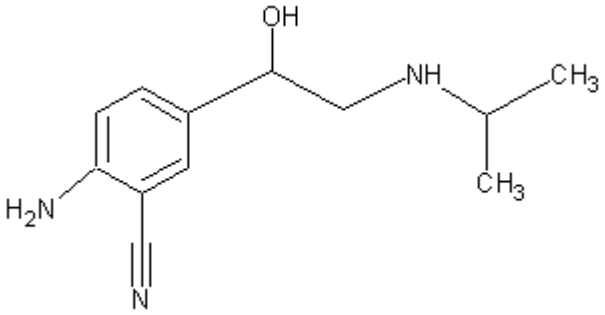
3. Structures



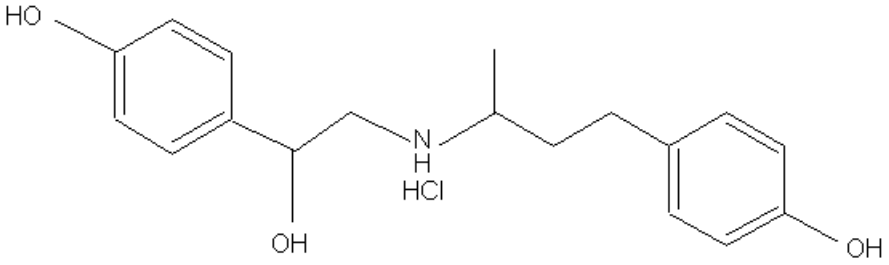
a. Clenbuterol ● HCl



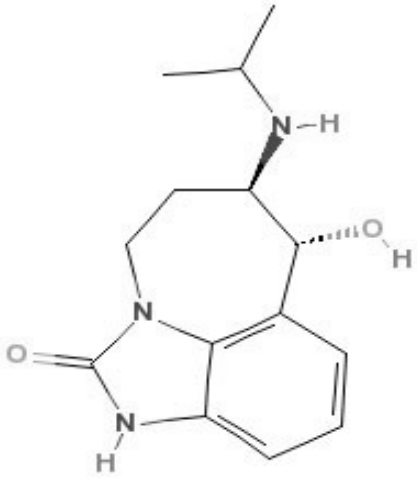
b. Salbutamol



c. Cimaterol



d. Ractopamine ● HCl



e. Zilpaterol

K. APPROVALS AND AUTHORITIES

1. Approvals on file.

   a. Issuing Authority: Director, Laboratory Quality Assurance Staff.