

Salbutamol (Albuterol): Detection and Confirmation

(With a modified SPE Procedure For B₂-Agonists In Equine Urine)

Developed By

The PA Equine Toxicology & Research Center
(Attn.: Dr. Uboh (610) 436-3501; Fax (610) 436-3504)

Drug Administration Facility:
University of Pennsylvania, New Bolton Center
(Attn.: Dr. Soma (610 444-5800 ext 2265; Fax (610) 444-4724)

For Testing Integrity Program

Principles:

Salbutamol is a bronchodilator often administered as an aerosol to race horses. It is detected by ELISA using a generic bronchodilator test kit (Elisa Technologies) and confirmed by GC/MS after sample cleanup with Solid Phase Extraction (SPE) procedures. In our hands, we have found this method of Solid Phase Extraction to be useful in the extraction of Salbutamol, Mabuterol, Clenbuterol, Metaproterenol, and Terbutaline from the equine urine.

Safety Precautions

General Good Laboratory Practices

Scope

The following SOP is proposed for detection by ELISA and GC/MS confirmation of salbutamol in equine urine to a concentration of **10 nanogram/mL** urine.

Definitions

GC/MS: Gas Chromatography with Mass Spectrometry
PETRL: PA Equine Toxicology & Research Laboratory
SOP: Standard Operating Procedure
TMS: Trimethylsilyl
BSTFA: bis(trimethylsilyl)trifluoroacetamide
SPE: Solid Phase Extraction
ELISA: Enzyme Linked Immuno-Sorbent Assay
PC: Positive Control
NC: Negative Control

Detection:-

By **ELISA** using Generic Bronchodilator test kit (PETRL SOP # 151) by Elisa Technologies.

Reagent Concentrations:

Concentrated enzyme diluted 1:90 with enzyme diluent (EIA buffer for ELISA Technologies),
25 µl of each sample and control were analyzed
100 µl enzyme conjugate added to each well
150 µl of K-Blue substrate (ELISA Technologies) was added

Note: Initial (+)'s on the ELISA Bronchodilator kit should be subjected to the additional screens detailed in the "Bronchodilator ELISA Flow Scheme" to further predict the suspect analyte.

GC/MS Prep:

EH/SPE: 3x5 mL aliquots of each administration urine sample are subjected to enzyme hydrolysis (EH) followed by solid phase extraction (SPE)

Pretreatment of Samples: Enzyme Hydrolysis

1. Label 16x150 mm screw-top test tubes to accommodate 3 aliquots of each administration sample plus QA samples (normally one PC, 25 ng/ml of salbutamol, and one NC, negative urine).
2. Label for each tube in step #1 a corresponding 16x125 mm culture tube for sample transfer.
3. To each screw-top tube add 4 mL pH 4.5 phosphate buffer.
4. To each screw-top tube add 1.2 mL enzyme solution .
5. Add 5 mL urine to corresponding screw-top tube from each urine sample.
6. Cap each tube and rock tube back and forth once to mix contents by inversion.
7. Place screw-top tubes in 65°C water bath for three hours.
8. Remove from water bath and cool by immersion in cold tap water in a sink.

Solid Phase Extraction Procedures (SPE)

- (1) Following the EH treatment, the salbutamol administration aliquots were pooled and the pH of each group of samples was adjusted to 6.00 using 50% KOH or 10% HCl as necessary.
- (2) The pooled administration urine, the negative and positive control samples (all at pH 6.00) were then centrifuged at 27,500 rpm for 50 minutes (Sorvall R-28 High Speed).
- (3) The supernatant from each sample was recovered by decantation.
- (4) As many 9.8 mL aliquots of each administration sample supernatant fractions as possible were put into 16 x 100 mm test tubes (Fisher brand Catalog #14-961-29) for SPE on the Benchmate workstation. A single tube of 9.8 mL each for the positive control and the negative control samples was subjected to the solid phase extraction.

Please note: the volume of the sample will increase from 5.0 mL to well over 10 mL after enzyme hydrolysis and dilution with phosphate buffer.

note: if the urine sample is viscous, a dilution of at least 1:3 of the urine with potassium phosphate buffer (0.1 M; pH 6.00) will be necessary before adjusting the final pH to 6.00. If filtration step is included, a 1:1 dilution of a “normal” consistency of the urine would still be necessary to minimize clogging of the filter in the SPE procedure.

Automatic Benchmate Procedure (Zymark):

Program: Pre-wet the filter (Whatman Syringe Filter, 0.45 micron PVDF disc) with 0.1 mL water and then filter to collect the sample. Rinse the filter holder with 2.0 mL methanol (HPLC grade). The column used was Bond Elut Certify (3 mL/300 mg; Part #1210-2081) by Varian.

The column was sequentially conditioned with 4 mL each of methanol and potassium phosphate buffer (0.1M; pH 6.00) and the sample (9.8 mL) was loaded onto the column and collected to waste. Thereafter, the column was sequentially rinsed with 2.0 mL acetic acid (1.0 M) and 3.0 mL water (HPLC grade). The column was then dried under compressed air for 300 seconds before it was again rinsed with 3.0 mL methanol. Following the methanol rinse, the column was again dried for 300 seconds under compressed air. The analyte was eluted with 5 mL of freshly prepared ethyl acetate and ammonia (50:1). The syringe was washed with 3.0 mL methanol after each extraction procedure was completed. Note that the extra wash and dry steps result in very clean samples.. (Please see the attached sheet titled “Zymark Benchmate 3.0 for setup Parameters 1 and 2.)

Alternative SPE Method: Manual SPE Procedure (using the Vac Elute Sps 24 By Analytichem Int'l):

When using the manifold setup, it is important to note that the steps involving the loading of the sample and the elution of the analyte should be performed at between 1 and 2 psi. Conditioning of the column should be carried out at 5 psi whereas drying of the column should be performed at above 15 psi and for longer than 300 seconds.

Confirmation Of Salbutamol By GC-MS:

Limit = **10 nanograms/milliliter** of urine sample.

Derivatization: Briefly, to the dried eluent obtained from the Solid Phase Extractions prepared from each time period collection, a 30 µL volume of BSTFA (O-bis trimethylsilyl)trifluoroacetamide) is added, the tubes are capped and heated at 65°C for 30 minutes. The reagent is evaporated under a gentle stream of nitrogen and 50 µL ethyl acetate is added. A 3 µL aliquot of each BSTFA-derivatized sample is injected via splitless injection. Salbutamol was detected through 8-hour post administration but was confirmed through 6-hour sample, post administration..

GC/MS Conditions:

Column: Capillary - 25mm, BPX-5, 0.2 μ (SGE)

Head Pressure: 8 psi

Initial Temp: 65°C 1 min. hold

Program Rate: 30°C/min.

Final Temp: 320°C, 3.5 min. hold

GC-MS run was performed in full scan EI mode over the mass range of 46-446 amu
(Base Peak = 369)

Reagents for Salbutamol SOP

Enzyme Hydrolysis:

Formula #48. Ammonium Hydroxide (NH₄OH):DI H₂O (1:1) Reagent For Use in EH Urine Extraction

Procedure (perform under fume hood):

Combine 500 mL concentrated NH₄OH and 500 mL DI H₂O in an Oxford re-pipettor bottle.

Formula #49. Ascorbic Acid solution 10% for Use in Enzyme Hydrolysis Urine Extraction

Procedure for 1 Liter:

Dissolve 100 gm of L-Ascorbic Acid (Fisher) into 1 liter of DI H₂O.

Formula #50. β-Glucuronidase (*Patella vulgata*) Enzyme Used for Enzyme Hydrolysis Urine Extraction

Procedure: For a minimum final concentration of 5000 AU/mL

1. 1 vial 500,000 units in 88 mL DI H₂O.
2. 1 vial 1,000,000 units in 175 mL DI H₂O
3. 1 vial 2,000,000 units in 350 mL DI H₂O
4. Use 1 1/2 mL per sample.
5. Store at 4° C (Good for 4 to 6 days).

Formula #52. DCM:IPA (10:1) Reagent For Use in EH Urine Extraction. Procedure for 4000 mL:

1. From a fresh 4000 mL bottle of dichloromethane (DCM) remove 500 mL DCM (place 3500 mL DCM in DCM pipettor bottle).
2. Add 350 mL isopropanol (IPA) and **mix thoroughly**.

Formula #59. pH 4.5 Buffer For Use with Enzyme Hydrolysis Urine Extraction.

Procedure for 1 liter:

1. Prepare a saturated solution of monobasic potassium phosphate (KH₂PO₄) by adding KH₂PO₄ to 1 liter of DI H₂O while stirring until saturated (no more will go into solution) and a precipitate remains. The pH of this solution should be 4.5 if it is saturated.
2. Let stand a minimum of 12 hours and decant clear solution into a clean reagent bottle.

Formula #69. Sulfuric Acid (1N(0.5M) H₂SO₄) For Use in EH Urine Extraction

Procedure for 3600 mL. (Wear goggles.):

1. Pour 2000 mL of DI h₂O into a 4000 mL flask.
2. Slowly add 100 mL of Conc. H₂so₄ (36N)/mix thoroughly while you add.
3. Dilute to 3600 mL w/ DI h₂O.

Warning: Add acid to H₂O. **Never** add water to acid

Solid Phase Extraction:

- **Potassium Phosphate** 0.1M, pH 6 (1 Liter)

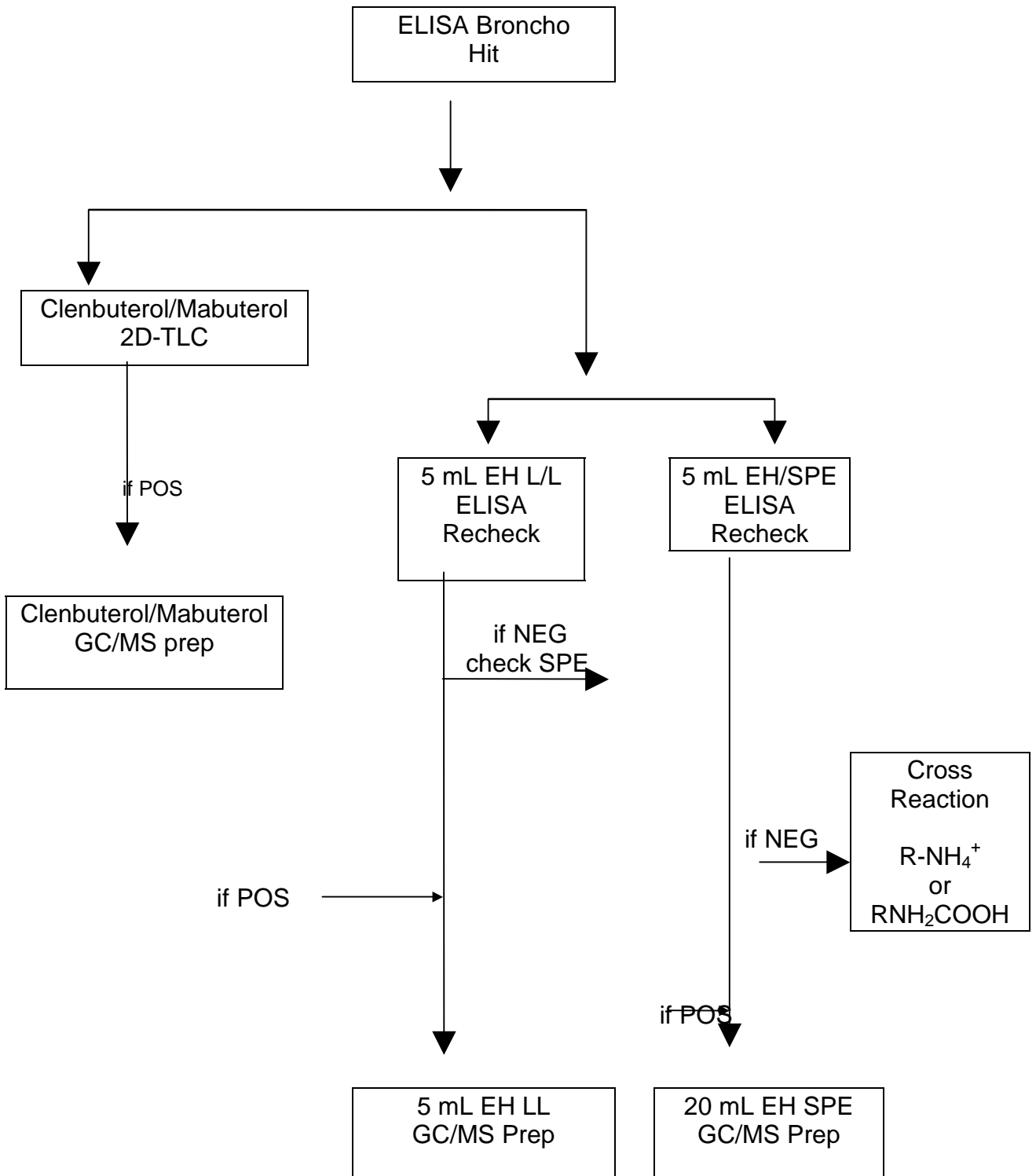
Weigh 13.61 g of KH₂PO₄ (MW 136.09) into a 1 Liter volumetric flask. Dissolve the KH₂PO₄ into 900 mL DI H₂O. Adjust pH to 6.0 with 1.0 M potassium hydroxide. Bring to 1 Liter volume. Good for 30 days.

- **MeOH**
- **Acetic Acid** 1 M
- **Elution Solvent: Ethyl Acetate: Ammonia** (50:1) made fresh each use

GC/MS

BSTFA: bis(trimethylsilyl)trifluoroacetamide (Pierce)

Flow Chart for Bronchodilator ELISA Positives Towards GC/MS Preps



**Addendum to TIP Salbutamol (Albuterol) SOP
November 4, 1996**

The PETRL offers these additional suggestions to increase the ruggedness and sensitivity of the TIP Salbutamol SOP:

Solid Phase Extraction:

1. The Solid Phase prep is crucial to recovery and confirmation of low levels of salbutamol
2. The same columns (Bond Elute Certify, part #1210-2081, 3 mL/300 mg) by Varian MUST be used.
3. ALL SPE reagents must be fresh (i.e. no more than one week old)
4. Elution solvent (Ethyl Acetate:Ammonia 50:1) MUST be **made fresh each day**
5. Centrifugation of the sample before SPE as per the SOP is also crucial to remove the sediment normally present in equine urine (for those without a high speed centrifuge, 3000 rpm works almost as well)
6. The pH values as detailed in the SOP are also crucial