

# **Metoprolol: Detection and Confirmation**

## **A Procedure Developed By**

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## **For Testing Integrity Program**

**(Dedicated to Dr. Robert Jack)**

### **Safety Precautions**

Solvent tanks for TLC plates and spray procedures must be performed in a fume hood. Safety goggles should be worn.

### **Scope**

The following SOP is proposed for detection and confirmation of metoprolol in equine urine to a concentration of 100 ng/mL urine.

### **Definitions**

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

### **Detection: By ELISA**

Using Generic Bronchodilator 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 from ELISA Technologies

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

## **Detection: By TLC**

5 mL enzyme hydrolysis followed by liquid/liquid extraction (PETRL SOP #107):

**Basic Principles:** Specific enzyme used to cleave  $\beta$ -Glucuronide linkages. Liquid/liquid extraction at proper pH range to yield desired drugs. Back extraction to yield cleaner extract.

### **Reagents**

pH 4.5 Phosphate Buffer — Formula #59  
 $\beta$ -Glucuronidase Enzyme Solution — Formula #50  
Ascorbic Acid Solution (10%) — Formula #49  
Ammonium Hydroxide:DI Water (50:50) — Formula #48  
DCM:IPA (10:1) — Formula #52  
Sulfuric Acid 0.5M — Formula #69  
1000:15 --(methanol: ammonia) solvent system  
Fast Black overspray

### **Extraction:**

1. Label sufficient number of 16x150 mm screw-top test tubes to accommodate the samples plus QA samples (normally one PC and one NC).
2. Label for each tube in step #1 a corresponding 16x125 mm culture tube for pour-over.
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 cool tap water in sink.
9. Uncap tubes and add 0.5 mL of 10% ascorbic acid solution to each tube.
10. Add that amount of ammonium hydroxide:water (50:50) to effect final pH of 10.0  $\pm$  1 pH unit.
11. Add 5 mL DCM:IPA (10:1) to each tube.
12. Cap tubes and rotorack for 5 minutes.
13. Centrifuge for 5 minutes at 3/4 speed setting.
14. Uncap tubes and aspirate upper, aqueous layer to waste.
15. Add 3 mL 0.5 M sulfuric acid (formula #69) solution to each tube.
16. Recap tubes and rotorack for 5 minutes.
17. Centrifuge for 5 minutes at 3/4 speed setting.
18. Uncap and working quickly, aspirate the lower, organic layer to waste
19. Add 0.2 mL of 10% ascorbic acid solution to each tube.
20. Add that amount of ammonium hydroxide:water (50:50) to effect final pH of 10.0  $\pm$  1 pH unit. Use pH meter to check.
21. Add 5 mL DCM:IPA (10:1)
22. Recap all tubes and rotorack for 5 minutes.
23. Centrifuge for 5 minutes at 3/4 speed setting.
24. Uncap tubes and aspirate upper, aqueous layer to waste
25. Pour remaining organic layer into corresponding 16X125 mm culture tube.
26. Dry in 65°C water bath in fume hood.

### **TLC Analysis**

1. Reconstitute the contents of each dried EH extract tube in 10  $\mu$ l DCM and vortex
2. Spot the entire contents of the tube on the 1 cm line of the TLC plate
3. Spot standards as required per QA.
4. Develop the plate to 5 cm solvent line in 1000:15 ("T-1"; Methanol: Ammonium Hydroxide) solvent system in a fume hood.
5. Dry plates in fume hood with hair dryer.
6. Spray plates lightly with Fast Black overspray and wait 15 minutes.
7. Metoprolol will appear as an orange spot with an  $R_f$  of approximately 5.6

### **Confirmation:- By GC/MS**

**Extraction:** 5 mL enzyme hydrolysis followed by LL extraction (see above)

**Derivatization:** The dried EH extraction is treated with 20 $\mu$ l BSTFA (Pierce) for 15 minutes at 80°C in a capped glass tube then 2  $\mu$ l are injected for GC/MS analysis.

#### **GC/MS Conditions:**

Column: SGE 25 m BPX-5 (21 mm ID)

Head Pressure: 8 psi

Initial Temp: 65°C 1 min. hold

Rate: 30°C/min.

Final Temp: 320°C 3.5 min. hold

Monitor ion 72 for metoprolol and its metabolites. Individual ions of metabolites can be distinguished from the accompanying mass spectra data.

## Reagents for Metoprolol SOP

**Formula #48.** Ammonium Hydroxide (NH<sub>4</sub>OH):DI H<sub>2</sub>O (1:1) Reagent For Use in EH Urine Extraction

Procedure (perform under hood):

Combine 500 mL concentrated NH<sub>4</sub>OH and 500 mL DI H<sub>2</sub>O in an Oxford re-Pipetter 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 H<sub>2</sub>O.

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

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

1. 1 vial 500,000 units in 88 mL DI H<sub>2</sub>O.
2. 1 vial 1,000,000 units in 175 mL DI H<sub>2</sub>O
3. 1 vial 2,000,000 units in 350 mL DI H<sub>2</sub>O
4. Use 1 1/2 mL per sample.
5. Store at 4 °C. Maximum shelf life is 6 days if enzyme solution has been stored at 4°C (less, if left at room temperature for several hours).

**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 in DCM Pipetter bottle).
2. Add 350 mL isopropanol (IPA) and **mix thoroughly**.

**Formula #59.** pH 4.5 Buffer For Use with Enzyme Hydrolysis Urine Extract TLC Plates

Procedure for 1 liter:

1. Prepare a saturated solution of monobasic potassium phosphate (KH<sub>2</sub>PO<sub>4</sub>) by adding KH<sub>2</sub>PO<sub>4</sub> to 1 liter of DI H<sub>2</sub>O while stirring until 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) H<sub>2</sub>SO<sub>4</sub> For Use in EH Urine Extraction

Procedure for 3600 mL. (Wear goggles.):

1. Pour 2000 mL of DI h<sub>2</sub>O into a 4000 mL flask.
2. Slowly add 100 mL of Conc. H<sub>2</sub>so<sub>4</sub> (36N)/mix thoroughly while you add.
3. Dilute to 3600 mL w/ DI h<sub>2</sub>O.

**Warning:** Add acid to H<sub>2</sub>O. **Never** add water to acid

**Fast Black overspray:** (Dissolve 50 mg Fast Black (Sigma) in 50 mL DI water, add 150 mL methanol)

## Flow Chart for Recheck of Bronchodilator ELISA Positives GC/MS Preps

