

# **Nabumetone (Relafen<sup>®</sup>): Detection and Confirmation of the Metabolite 6-MNA**

A Procedure Developed  
By

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## **For Testing Integrity Program**

### **Principles**

Nabumetone (Relafen<sup>®</sup>) is a non-steroidal anti-inflammatory pro-drug (NSAID). The pharmacologically active metabolite is 6-methoxy-naphthyl-acetic acid (6-MNA). By virtue of nabumetone being a pro-drug, very little of it is excreted unchanged in horse urine following administration, which explains why nabumetone is not detected in the urine. Although several metabolites are readily detected in post administration samples, there is currently a traceable standard for only one: the 6-MNA (obtained for research purposes from Smith Klein Beecham Pharmaceuticals). Therefore, this SOP is targeted at 6-MNA for detection and confirmation in the equine urine.

### **Safety Precautions**

Solvent tanks for TLC plates and spray procedures must be performed under a fume hood. Safety goggles should be worn. Reagents containing organic solvents should be prepared under a fume hood.

**Caution** should be used when preparing Liebermann's Overspray, as considerable levels of nitrous fumes and heat are evolved!

### **Scope**

The following SOP is proposed for TLC detection and GC/MS confirmation of 6-MNA in equine urine to a concentration of **1 microgram/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

L/L: liquid/liquid

TMS: Trimethylsilyl

BSTFA: bis(trimethylsilyl)trifluoroacetamide

AU Acid Urine Extraction

PC: Positive Control

NC: Positive Control

BH: Base Hydrolysis

## **Detection By TLC: Base Hydrolysis/Acid Extraction (BH/AU)**

### **Principle:**

Base hydrolysis aids in the release of drugs which have been conjugated by the horse. A petroleum ether-dichloromethane acid extract is then performed to extract the nabumetone metabolites from the horse urine.

### **Reagents**

- 0.93 N sodium hydroxide — Formula #64
- Saturated phosphate buffer pH 4.5 — Formula #59
- pH 2.0 buffer — Formula #58
- Petroleum ether(pet ether)
- Dichloromethane(DCM)
- 90:25:2 solvent system
- Liebermann's spray reagent — Formula #36
- Ehrlich's (modified) spray reagent — Formula #27
- Concentrated hydrochloric acid

### **Extraction Procedure:**

#### ***Base Hydrolysis***

1. Label a sufficient number of 16 x 125 screw-top test tubes to accommodate the # of samples to be extracted and any appropriate PC's and NC's.
2. Label sufficient number of 16 x 125 culture tubes for use as pour over tubes for each tube in step 1.
3. To each screw top tube add 2 mL of urine.
4. To each sample add 1.0 mL of 0.93 N Sodium Hydroxide. Vortex for 30 seconds, allow mixture to stand at room temperature for 10 minutes.
5. Add 3 mL of pH 2.0 buffer to each tube.
6. Add 2 mL of pH 4.5 buffer.
7. pH sample to 3.0-3.5 using pH 2.0 buffer. **Caution: Some compounds will not be extracted if the sample is not acidic enough.**
8. Add 4 mL of Pet ether and 1 mL of DCM to each tube.
9. Cap each tube and rotorack for 5 minutes.
10. Centrifuge @ 3000 rpm for 5 minutes.
11. **Aspirate the bottom** (aqueous) layer to waste.
12. Transfer the **remaining** liquid (solvent) to a clean, pre-labeled, 16 x 125 culture tube.

13. Evaporate to dryness in a 60°C water bath.
14. Dissolve residue in 2-4 µl of DCM.

## **TLC Analysis**

### ***1. Prepare 1-Dimensional (1-D) TLC Plates as follows***

- A. Using the plate cutter or scribing tool, cut the 20x20 cm plate in half, resulting in two 10 x 20 cm TLC plates.
- B. Using a sharp point and a metal ruler, scribe a line through the silica to the glass **exactly** 5 cm from one of the long sides.
- C. Using a soft (#2) lead pencil and the metal ruler, lightly draw a line 1 cm from the base of one of the long sides.
- D. Store the cut and scored plates in a warm oven to prevent absorption of moisture.

### ***2. Spot plate:***

1. Add 10 µl of DCM to evaporated BH extracted tube contents and vortex.
2. Spot 1 Merck 60F-254 TLC plate. Spot 1/3 of the total of each sample on the plate.
3. Spot standards as required per QA as well as a 6-MNA standard.
4. Develop plate to the ascribed 5 cm solvent front in 90:25:2 solvent system in a fume hood.
5. Dry plate in fume hood with hair dryer.

### ***3. Spray Plate***

1. Spray plate with Modified Ehrlich's reagent.
2. Spray plate with Concentrated hydrochloric acid
3. Gently heat on hot plate, then allow to cool
4. Spray plate with Liebermann's reagent.

The reference standard (6-MNA) as well as positive samples yield a greenish black spot at  $R_f$  of 5.6

## **Alternate TLC Procedure: Enzyme Hydrolysis/Acid Extraction (EH/AU)**

As an alternate screening technique, 100 µl urine subjected to enzyme hydrolysis, followed by acid liquid/liquid extraction gives a higher total yield of all the nabumetone metabolites and thus may also be employed as a screen for the compound. This method is detailed as follows.

## **Principle**

Specific enzyme used to cleave β-Glucuronide linkages. Liquid/liquid extraction at proper pH range to yield desired drug analytes.

## **Reagents**

β-Glucuronidase Enzyme Solution — Formula #50  
pH 2.0 buffer — Formula #58  
pH 4.5 Phosphate Buffer — Formula #59

90:25:2 (toluene: dioxane: glacial acetic acid) solvent system  
Fast Blue B TLC over spray Formula #28  
Liebermann's Reagent over spray Formula #36  
Dichloromethane (DCM),

## **Extraction Procedure:**

### ***Enzyme Hydrolysis***

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 100 µl 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 a sink.

### ***Acid Urine Extraction***

1. Label a 16 mm x 125 mm ***screw-top*** disposable borosilicate glass culture tube and a 16 mm x 125 mm standard disposable borosilicate culture tube for each sample hydrolyzed above for pour-over
2. To each screw-top tube, add the following:
  - 3 mL pH 2 buffer (Formula #58)
  - 5 mL methylene chloride (DCM), ACS grade to the entire contents of the enzyme hydrolyzed sample tube
3. Cap all screw-top tubes tightly and rack tubes for 5 minutes.
4. Centrifuge all tubes for 5 minutes at 3000 rpm.
5. Remove tubes and carefully aspirate off the top (aqueous) layer to waste.
6. Decant the remaining DCM into the corresponding clean tube designated for pour-over, being careful NOT to contaminate the sample with any biological or aqueous material remaining from the aspiration step.
7. Bring tubes from step #6 above to dryness by placing all tubes into a 65°C water bath and allowing the DCM to evaporate completely. Use a water bath under a fume hood.
8. Remove tubes from water bath, place in rack, and allow to cool to room temperature.

## **TLC Analysis :**

1. Reconstitute the contents of each dried EH/AU extract tube in 10 µl 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 90:25:2 (toluene: dioxane: glacial acetic acid) solvent system in a fume hood.
5. Dry plates in fume hood with hair dryer.

6. Spray plates lightly with Liebermann's Reagent and heat.
7. 6-MNA will appear as a gray-green spot with an  $R_f$  of approximately 5.6 after the Liebermann's spray and turns black upon heating.
8. Alternatively, several of the conjugate metabolites can be visualized with Fast Blue B overspray on a second plate if desired.

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

### **Principles**

Tri methylsilyl (BSTFA) derivatives were employed to produce a better chromatographic separation between the methoxy and corresponding hydroxy metabolites. See the accompanying GC/MS data package for discussion of the various proposed metabolites detected by GC/MS from an administration of Nabumatone.

**Extraction:** Enzyme hydrolysis of 100  $\mu$ l urine followed by AU L/L extraction (see above)

**Derivatization:** The dried EH/AU extract is treated with 50  $\mu$ l BSTFA (Pierce) for 30 minutes at 60°C in a capped glass tube. The reagent is dried under a gentle stream of nitrogen and 50  $\mu$ l ethyl acetate is added; 2  $\mu$ l are then injected for GC/MS analysis.

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

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

Head Pressure: 8 psi

Initial Temp: 65°C 1 min. hold

Program Rate: 20°C/min.

Final Temp: 320°C 5 min. hold

Monitor ion 171 for 6-MNA. Other relevant ions can be determined from the enclosed GC/MS data package.

## Reagents for Nabumetone SOP

### **Formula #50.** $\beta$ -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 (Prepare only enough for 4-6 days at one time).

### **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 (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 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.

### **90:25:2 TLC solvent system** (Toluene:Dioxane:Glacial Acetic Acid)

#### **Formula #28 — Fast Blue B Overspray.**

Procedure for 200 mL

1. Dissolve 50 mg of Fast Blue B Salt (o-Dianisidine, tetrazotized) in 50 mL DI H<sub>2</sub>O.
2. Add 150 mL Methanol.

#### **Formula #36 — Liebermann's Overspray**

Procedure for 800 mL:

1. **Caution!** Considerable levels of nitrous fumes and heat are evolved. Reaction must be carried out in a fume hood.
2. Dissolve, a little at a time (very little), 80 gm potassium nitrite (KNO<sub>2</sub>) in 800 mL conc. H<sub>2</sub>SO<sub>4</sub>.

#### **Formula #27 — Ehrlich's (Modified) Overspray**

Procedure for 480 mL spray:

1. Dissolve 5 gm p-dimethyl-aminocinnamaldehyde (kept in freezer) into 475 mL DI H<sub>2</sub>O.
2. Add 25 mL conc. HCl. (Do in fume hood.)
3. Mix solution until #1 is completely dissolved.

#### **Formula #58 — pH 2 Buffer For Use in Acid Urine Extraction**

Procedure for approximately 800 mL:

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> crystals to 800 mL DI H<sub>2</sub>O until no more will go into solution and a precipitate remains. Let stand a minimum of 12 hours.
2. Decant by pouring saturated solution into a clean reagent bottle and pH to 2 with conc. phosphoric acid (H<sub>3</sub>PO<sub>4</sub>).

#### **Formulas #64 —0.93 N Sodium Hydroxide (NaOH)**

Dissolve 37.2 gm sodium hydroxide in 1000 mL of DI H<sub>2</sub>O

## **References**

- (1) Metabolism of nabumetone (BRL 14777) by various species including man: Haddock et al, *Xenobiotica*, 1984, 14 (4): 327-337.
- (2) Disposition and excretion of 6-methoxy-naphthylacetic acid, the active metabolite of nabumetone in horses: Soma et al, *AJVR*, 1996, 57 (4): 517-521