

# **Detection and Confirmation of Oxaprozin and its Metabolites in Equine Urine**

A Procedure Developed at

The Drug Testing Laboratory  
Texas Veterinary Medical Diagnostic Laboratory  
College Station, TX 77843

Allen C. Ray, M. M. Rao, Kenneth Peck

Phone: 409-845-3414 or 9011

Fax: 409-845-1794

e-mail: [ac-ray@tvmdl.tamu.edu](mailto:ac-ray@tvmdl.tamu.edu)

For

The Testing Integrity Program

1999

## Oxaprozin:

### Detection and Confirmation of Oxaprozin and its Metabolites in Equine Urine

#### **Abstract:**

Oxaprozin (Daypro, Wyeth-Ayerst), a non-steroidal anti-inflammatory drug, was administered to a horse as a single oral dose of 2400 mg (maximum single dose for humans is 1200 mg). Urine was collected at 0, 6, 10, 30, and 50 hours. Oxaprozin and metabolites were detected by TLC at 6 and 10 hours after SPE (acid fraction, DCM with 5% MeOH, followed by back-extraction). The primary metabolite was more easily observed by TLC and detected through 30 hours. The limit of detection for parent oxaprozin by TLC was approximately 1000 ng/ml in equine urine. Abbott's benzodiazepine fluorescent polarization reagent cross-reacts with parent oxaprozin at approximately 2 – 4%. No reaction was observed with the benzodiazepine ELISA kit (Neogen). GC/MS analysis of SPE and L/L (BH/AU) extracts confirmed the presence of oxaprozin and two hydroxylated metabolites through 50 hours as the TMS-derivatives. Although parent oxaprozin was observed, the most abundant product was a hydroxylated metabolite with the hydroxyl group probably present on one of the aromatic rings (reference standard was not available for comparison). Another monohydroxy metabolite and a trace of the di-hydroxy metabolite were also detected. Enzyme ( $\beta$ -glucuronidase) hydrolysis of urine prior to extraction did not increase yields of oxaprozin and its metabolites.

#### **Safety Precautions:**

TLC procedures must be performed in a fume hood. Protective clothing (safety goggles, lab coat, and gloves) should be worn. Consult the material safety data sheets for unfamiliar chemicals used in this procedure.

#### **Scope:**

This SOP describes a method for TLC detection and GC/MS confirmation of oxaprozin and its metabolites in equine urine.

#### **References:**

Janssen, FW, et al: Metabolism and Kinetics of Oxaprozin in Normal Subjects. Clin Pharm Therap, 27 (3): 352 – 62 (1980).

Fraser, AD, Howell, P: Oxaprozin Cross-Reactivity in Three Commercial Immunoassays for Benzodiazepines in Urine. J Analyt Tox, 22 (1): 50 – 4 (1998)

### **Definitions:**

TLC – Thin Layer Chromatography  
GC/MS – Gas Chromatography/Mass Spectrometry  
SOP – Standard Operating Procedure  
L/L – Liquid/Liquid Extraction  
TMS – Trimethylsilyl  
DI-H<sub>2</sub>O – Deionized Water  
BSTFA – bis(trimethylsilyl)trifluoroacetamide  
PC – Positive Control Urine  
NC – Negative Control Urine  
SPE – Solid Phase Extraction  
BH – Base Hydrolysis  
AU – Acid Urine Extract  
ELISA – Enzyme-linked ImmunoSorbent Assay  
UCT – United Chemical Technologies  
PC – Positive Control  
NC – Negative Control  
HOAc – Acetic Acid

### **Principle:**

Oxaprozin and metabolites can be detected using TLC after removal of endogenous urine components by SPE and further L/L back extraction. Though somewhat cumbersome, this technique is recommended to remove background substances, which interfere with TLC visualization. However, simple BH/AU L/L extraction is adequate for GC/MS confirmation.

### **Standards:**

Oxaprozin pure drug standard was generously donated by Wyeth-Ayerst. Metabolite standards were not available. A 1 mg/ml standard solution of oxaprozin prepared in methanol was used in the analysis and stored at -20°C until needed for analysis.

### **Reagents:**

Phosphate Buffer (saturated) pH 3.3 (AU)

Pour potassium phosphate monobasic (KH<sub>2</sub>PO<sub>4</sub>) into approximately 500 ml DI-H<sub>2</sub>O until the solution is saturated (if all the crystals or powder dissolve, solution is not saturated). Transfer supernatant to a beaker and adjust pH to 3.3 using concentrated phosphoric acid.

1.0 N Acetic Acid (HOAc) for SPE

Add 201 ml glacial acetic acid (CH<sub>3</sub>CO<sub>2</sub>H) to 3299 ml deionized water.

#### 0.2 M Phosphate Buffer pH 6.5 [SPE]

Dissolve 87.04 g potassium phosphate monobasic ( $\text{KH}_2\text{PO}_4$ ) in 3200 ml deionized water. Mix well. Adjust pH to 6.5 with sodium hydroxide or potassium hydroxide pellets (approximately 11 g) or 5 – 10 N NaOH or KOH.

#### Sodium Carbonate ( $\text{Na}_2\text{CO}_3$ ) Buffer 1 M

Dissolve 106 g sodium carbonate ( $\text{Na}_2\text{CO}_3$ ) in 1000 ml deionized water. Transfer solution into an oxford pipettor.

#### Sodium Hydroxide (NaOH) 0.1 N (AU)

Dissolve 4.0 g sodium hydroxide (NaOH) pellets into 1000 ml deionized water. Transfer solution into an oxford pipettor.

#### 75:21:4 (AU)

Thoroughly mix 75 ml toluene, 21 ml dioxane, and 4 ml acetic acid. Transfer mixture into TLC tank, cover, and allow to equilibrate for 15 minutes.

#### 4:4:2 (AU)

Solvent: chloroform 40 ml : cyclohexane 40 ml : acetic acid 20 ml  
Stock: chloroform : cyclohexane (2000 ml : 2000 ml) : Mix stock solution well before dispensing. Dispense 80 ml stock solution into a 100- ml graduated cylinder. Add 20 ml glacial acetic acid. Transfer into TLC tank, cover, thoroughly mix by tilting tank, and allow to equilibrate for 15 minutes.

#### Dragendorff's Spray

Reagent A: Add 2.0 g bismuth subnitrate to 25 ml glacial acetic acid. Dilute to 100 ml with DI- $\text{H}_2\text{O}$ .

Reagent B: Dissolve 40.0 g potassium iodine in DI- $\text{H}_2\text{O}$ ; dilute to 100 ml with DI- $\text{H}_2\text{O}$

Spray: Mix 10 ml Reagent A and 10 ml Reagent B, add 20 ml glacial acetic acid; dilute to 100 ml with DI- $\text{H}_2\text{O}$ .

Unused A and B are stored at room temperature. Dragendorff's is stable for two days.

#### Sodium Nitrite Spray ( $\text{NaNO}_2$ )

Dissolve 5.0 g sodium nitrite in DI- $\text{H}_2\text{O}$ . Dilute to 100 ml with DI- $\text{H}_2\text{O}$ .

#### Cupric Chloride Spray ( $\text{CuCl}_2$ )

Dissolve 25 g of cupric chloride in 75 ml DI- $\text{H}_2\text{O}$  and 25 ml methanol mixture.

NOTE: Spray Reagents should be properly labeled with their identifying name, date of preparation and initials of tech preparing the solution. Expiration dates are generally not

applicable except for Dragendorff's (2 days). Appropriate expiration dates should be recorded on the label of this solution. Store extra spray solutions in the refrigerator.

**Apparatus:**

SPE manifold with trap  
16 x 125 mm screw-top glass tubes  
SPE columns (UCT mixed phase XTRACKT)  
Rotorack  
Centrifuge  
13 x 100 mm glass test tubes  
TLC Plates, 10 x 20 cm (Silica gel 60 FZ54, EM Science)  
10 µL Hamilton syringe or Eppendorf Ultra-micro pipettor.  
Vacuum source  
pH meter

**Sample Preparation:**

For TLC: Perform base hydrolysis of 5 ml urine prior to SPE.

For GC/MS: Perform BH/AU extraction or SPE prior to derivatization and GC/MS analysis.

**Controls Sample Preparation:**

Blank equine urine is used as a negative control. Equine urine spiked at 2 to 3 µg/ml of oxaprozin is recommended as a positive control.

**Solid Phase Extraction Procedure:**

NOTE: At this printing, the SPE columns in use were 500 mg mixed bed bonded silica (C-8 and sulfonic acid) proprietary columns (XTRACKT, #XRDAF515) by United Chemical Technologies, Bristol, PA. Expiration dates for these SPE columns are approximately two years after manufacture and should not be exceeded. In general, columns are used well prior to expiration dates. The protocol described below is for this product only, even though similar packings might involve basically identical protocols.

Sample Preparation

1. Add 1 ml of 0.1N NaOH (sodium hydroxide) to 5 ml of sample in a numbered 16 x 100 mm tube for the acid fraction – basic hydrolysis.  
Mix well and allow to stand at room temperature for 10 – 15 min.
2. Add 5 ml 0.2M Phosphate Buffer (pH 6.5) into the sample tube and mix.

3. Check the pH and, if necessary, add acid (6N HCl) or base (NH<sub>4</sub>OH:H<sub>2</sub>O, 50:50) until the pH is between 5.5 and 6.5. (Note: After each addition of acid or base, the sample must be mixed.)

### Column Preparation

All solid phase extraction manifolds are used according to manufacturer's instructions. Traps must be installed between manifold and vacuum source. A second trap containing copper turnings and cotton should be replaced when copper dulls.

1. Prepare numbered 16 x 100 mm disposable glass tubes labeled A (Acid/Neutral drug) for sample collection.
2. Place the appropriate number of stopcocks on the manifold.
3. Insert the appropriate number of SPE columns (numbered accordingly) on stopcocks.
4. Add 5 ml Methanol to each SPE column bed and aspirate slowly. Do not allow the column to go dry during steps 4 – 6. Use minimum vacuum ( $\leq 3$  in Hg).
5. Immediately add 5 ml DI-H<sub>2</sub>O to each column and aspirate.
6. Add 3 ml 1.0N Acetic Acid (HOAc) to each column and aspirate. At this time the column is activated.

### Sample Application, Washes, and Elution

NOTE: Viscous samples should be centrifuged prior to loading onto columns. If a sample does not flow through the column, it should be worked up individually. More vacuum may be applied or liquid/liquid protocols may be followed.

1. Apply the sample to the activated column at a rate of approximately 1 – 3 ml/min.
2. Add 5 ml 0.2 M Phosphate buffer (pH 6.5) to each column and aspirate.
3. Add 2 ml 1.0 N HOAc to each column and aspirate. Dispose of waste.
4. Dry the column under strong vacuum (e.g., 12 – 15 in Hg) for  $\approx 45$  – 60 min (or until dry).
5. Release vacuum.
6. Add 5 ml Hexanes to each column and aspirate. Dispose of waste.
7. Place numbered glass tubes labeled A in appropriate collection positions. Wash with 5 ml dichloromethane (DCM), ie discard.
8. Elute oxaprozin and metabolites with 5 ml DCM containing 5% methanol. Collect eluate at  $\leq 5$  ml/min. Use vacuum as necessary to recover residual solvent.
9. Remove the A tubes and place them in a test tube rack.
10. Add 2 ml of 1 M Na<sub>2</sub>CO<sub>3</sub>.
11. Tightly cap each tube and rotorack slowly for 5 minutes.
12. Aspirate bottom layer. SAVE TOP LAYER.
13. Adjust each tube to approximately pH 3.0 ( $\pm 0.3$ ) using 6N HCl (1 drop at a time), or phosphate buffer pH 3.3.
14. Add 5 ml DCM:pet ether (10:1) to each tube.

15. Tightly cap each tube and rotorack slowly for 5 minutes. (Centrifuge at 2000 – 2500 rpm to break emulsion, if necessary)
16. Aspirate off the top (aqueous) layer.
17. Evaporate solvent at  $40^{\circ}\text{C} \pm 5^{\circ}\text{C}$  with nitrogen ( $\text{N}_2$ ).

#### TLC Sample Application

1. Using a #1 pencil, lightly mark a line at 2 cm across a 10 x 20 cm TLC plate and heavily score a line at 7 cm. Above the 7 cm line, label the plate. Include date, technician's initials, race track, race date, solvent system, UV and spray sequences. Below the 2 cm line, label NC, PC, standards, and lab sample numbers at application spots. Place standards in the middle of the plate. Allow the plates to activate for at least 10 minutes on a hot plate (temperature  $113^{\circ}$  -  $130^{\circ}\text{F}$ ) before spotting.
2. Dissolve each sample in 20  $\mu\text{l}$  EtOAc immediately before spotting.
3. Spot 2  $\mu\text{l}$  of each sample, and 1  $\mu\text{L}$  of oxaprozin standard using either a 10  $\mu\text{l}$  Hamilton syringe or an Eppendorf Ultra-micro pipettor on each TLC plate.
4. Rinse the syringe with 30  $\mu\text{l}$  of ethyl acetate (EtOAc) between samples (pull up and dispense 10  $\mu\text{l}$  of EtOAc 3 times) or discard the used pipette tip.

#### TLC Plate Development

1. Develop TLC plate in 4:4:2, or 75:21:4 and remove plate from TLC tank when solvent front reaches 7 cm mark. While slightly damp, observe the plate under SUV (254 nm). Mark any quenching.
2. Spray with Dragendorff's, cover with a glass plate, and observe on light box.
3. Spray with  $\text{NaNO}_2$ , cover with a glass plate and observe on light box.
4. Spray with  $\text{CuCl}_2$ , cover with a glass plate, and observe on light box.
5. Oxaprozin and the major metabolite appear at  $R_f$  0.7 and  $R_f$  0.3 in 4:4:2 and at  $R_f$  0.5 and 0.35 in 75:21:4, respectively (spots turn brown after  $\text{NaNO}_2$  treatment and grey after  $\text{CuCl}_2$  treatment). The major metabolite spot is much more predominant than that of the parent compound. Quenching is an important indication of the presence of oxaprozin since blank urine may contain interfering co-extractives that react with  $\text{NaNO}_2$ .

#### **Alternate Liquid/Liquid Extraction Procedure:**

The base hydrolysis/acid urine extraction protocol is sufficient for GC/MS confirmation but contains numerous co-extractives, which interfere with TLC plate visualization.

1. To 2 ml of sample in a 16 x 125 mm screw-top glass tube, add 1 ml of 0.1N sodium hydroxide (NaOH) and allow it to remain at room temperature for 10 minutes.
2. Add 4 ml of saturated phosphate buffer pH 3.3.
3. Add 5 ml dichloromethane (DCM): petroleum ether (10:1) mixture.
4. Cap tightly and rotorack slowly for 5 minutes.
5. Centrifuge for 5 minutes at approximately 2000-2500 rpm.

6. Aspirate off the top aqueous layer.
7. Transfer the organic fraction to a clean disposable 13 x 100 mm glass test tube.
8. Evaporate at  $40^{\circ}\text{C} \pm 5^{\circ}\text{C}$  with nitrogen ( $\text{N}_2$ ).

### **Immunoassay Protocol:**

#### ELISA –

No appreciable cross-reactivity was observed with the benzodiazepine kit (Neogen) when equine urine spiked up to  $6\mu\text{g/ml}$  was assayed.

#### FPIA –

Some cross reactivity was observed with the benzodiazepine fluorescent polarization immunoassay (FPIA) reagent (Abbott). Urine spiked at 500 ng oxaprozin per ml registered 23 ng/ml by FPIA (4.6% cross-reactivity, nordiazepam calibrator); 1000 ng/ml registered 29 ng/ml (2.9%), and 2000 ng/ml read 41 ng/ml (2.1%).

The drug administration urine samples (6 – 30hr pooled) indicated a concentration of 126 ng/ml. Even though the low calibrator for this assay is 200 ng/ml, a reading of 126 ng/ml is well above background concentration.

### **GC/MS Confirmation Procedure:**

#### Derivatization protocol

1. Add 20 to 40  $\mu\text{l}$  commercially available BSTFA to the appropriate extraction residue in a small test tube or glass insert for an autosampler vial.
2. Heat for 1 to 2 min. at  $60^{\circ}$  -  $80^{\circ}\text{C}$  in a heating block (or equivalent). Longer heating times of up to 1 hour may be used without any observable difference.
3. One  $\mu\text{l}$  is injected for GC/MS analysis.

#### GC/MS conditions

Instrument HP 5972 MSD/5890 GC  
Column HP5 MS, 15m (0.25mm, 0.25 $\mu$ )  
Injection port temperature  $200^{\circ}\text{C}$   
Initial oven temperature  $80^{\circ}$  (1 min)  
Ramp rate  $20^{\circ}/\text{min}$   
Final oven temperature  $280^{\circ}\text{C}$  (10 min)  
Mass range 50 – 550 amu

#### Compounds identified and mass spectra

Oxaprozin TMS (RT=13.6 min) – m/z 248 ( $\text{M}^+ - 117$ ), 100%; 365 ( $\text{M}^+$ ), 18%; 350 ( $\text{M}^+ - 15$ ), 11%; 165, 17%

Hydroxyoxaprozin TMS (RT = 15.2 min, major metabolite) – m/z 336 ( $\text{M}^+ - 117$ ), 100%; 453 ( $\text{M}^+$ ), 30%; 438, 14%.

Hydroxyoxaprozin TMS (RT = 13.8 min, minor metabolite) – m/z 336 ( $M^+ - 117$ ), 100%; 453 ( $M^+$ ), 43%; 320, 17%; 438, 6%.

Dihydroxyoxaprozin diTMS (RT = 16.1 min, trace) – m/z 424 ( $M^+ - 117$ ), 100%; 541 ( $M^+$ ), 70%; 526, 22%; 336, 14%.

### **Quality Assurance:**

Critical Control Points -

As TMS derivatives are very sensitive to water, residues should be completely dry prior to the addition of BSTFA.

### **Summary:**

A hydroxylated metabolite of oxaprozin was the predominant compound detected in horse urine (reference standard was not available for comparative analysis) by both TLC and GC/MS analyses. Parent oxaprozin, another mono hydroxy metabolite and a dihydroxy metabolite were also observed in order of decreasing abundance. It is speculated that hydroxylation occurs on each of the aromatic rings, but this is yet to be confirmed.