

# **Carprofen: Detection and Confirmation**

Developed By

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## **For Testing Integrity Program**

## **STANDARD OPERATING PROCEDURE FOR:**

### **BASE HYDROLYSIS WITH ACID EXTRACTION PROCEDURE FOR CARPROFEN**

#### **METHOD REFERENCE**

NASRC Quality Assurance Program (1982-1988)

ARCI Quality Assurance Program (1988-current)

#### **SCOPE AND APPLICATION**

This technique can be applied to equine urine specimens.

Urine samples are hydrolyzed prior to extraction to release conjugated drugs and drug metabolites. A basic hydrolysis is performed to free small quantities of conjugated non-steroidal anti-inflammatory drugs such as carprofen. Conjugated metabolites of these drugs are also detected using this procedure.

The basic hydrolysis extract requires three separate TLC plates employing different developing and locating techniques.

#### **APPARATUS**

1. Science, silica gel, Fluorescent, Thin Layer Chromatography Plates
2. Capillary plate spotter
3. Hot plate with small fan
4. x 125 mm screw top test tubes with caps
5. Vortex mixer
6. Automatic pipettor with 10 ml graduated pipettes
7. Rotorack mixer for test tubes
8. Centrifuge
9. Vacuum aspiration apparatus
10. Disposable 15 x 85 mm tubes
11. Light box with 254 nm and 365 nm UV light
12. Water bath
13. Hot plate

#### **REAGENTS**

##### **Water**

- Use DI water in any reagent/procedure requiring the use of water.

##### **1 N Sodium Hydroxide**

#### Reagents

- sodium hydroxide, (NaOH) pellets
- water

#### Procedure

- dissolve 40.0 g of sodium hydroxide pellets in 1000 ml of water. Mix

### **pH 2.0 phosphate buffer**

*Prepare under a fume hood*

*Always add acid to water*

#### Reagents

- potassium phosphate monobasic ( $\text{KH}_2\text{PO}_4$ )
- water
- concentrated phosphoric acid

#### Procedure

- dissolve 880 g potassium phosphate in 4000 ml of water. Mix.
- to 3800 ml of the solution prepared above add 200 ml concentrated phosphoric acid. Mix.

### **Dichloromethane, reagent grade**

### **Dragendorff's solvent**

#### Reagents

- bismuth subnitrate
- water
- glacial acetic acid
- potassium iodide

#### Procedure

Mix equal parts of solution A and solution B.

#### Solution A

- Dissolve 9.4 grams of bismuth subnitrate in 600 ml of water.
- Add to the solution, 306 ml of glacial acetic acid.
- Bring to 1 liter total volume with water. Mix and filter
- Dilute to 100 ml with water. Mix.

#### Solution B

- Dissolve 112.1 grams of potassium iodide in 1000 ml of water.

### **Cupric chloride solution 25%**

#### Reagents

- cupric chloride
- methanol

#### Procedure

- Dissolve 200 g cupric chloride in 600 ml of water. Add 200 ml of methanol. Mix.

### **Mandelin's reagent**

#### Reagents

- ammonium meta-vanadate ( $\text{NH}_4\text{VO}_3$ )
- concentrated sulfuric acid ( $\text{H}_2\text{SO}_4$ )

#### Procedure

- Combine 0.5 g of ammonium meta-vanadate and 100 ml of concentrated sulfuric acid in a glass container.
- Shake vigorously and allow the solution to stand for 30 minutes.
- Decant the supernatant solution into a glass spray bottle. Use this for the overspray.

### **Sodium nitrite solution 5%**

#### Reagents

- sodium nitrite ( $\text{NaNO}_2$ ) crystals
- water

#### Procedure

- dissolve 5.0 g sodium nitrite in 100 ml of water. Mix.

### **Chloroform/cyclohexane/acetic acid (4:4:2)**

#### Reagents

- chloroform ( $\text{CHCl}_3$ )
- cyclohexane
- glacial acetic acid ( $\text{CH}_3\text{COOH}$ )

#### Procedure

- Combine 40 ml  $\text{CHCl}_3$ , 40 ml cyclohexane and 20 ml  $\text{CH}_3\text{COOH}$  using a labeled graduate cylinder.
- Pour the solvent (100 ml) into a developing tank.
- Immediately cover the tank with the glass lid.
- Gently rock the tank to mix the solvents.
- Allow the tank to equilibrate for 15 minutes before use.

### **Chloroform/ethanol (9:1)**

#### Reagents

- chloroform
- ethanol

#### Procedure

- Combine 90 ml  $\text{CHCl}_3$  and 10 ml ethanol using a labeled graduate cylinder.
- Pour the solvent (100 ml) into a developing tank.
- Immediately cover the tank with the glass lid.
- Gently rock the tank to mix the solvents.
- Allow the tank to equilibrate for 15 minutes before use.

### Davidow solvent

#### Reagents

- ethyl acetate (EtOAc)
- methanol
- concentrated ammonium hydroxide

#### Procedure

- Combine 85 ml ethyl acetate, 10 ml methanol and 5 ml ammonium hydroxide using a labeled graduate cylinder.
- Pour the solvent (100 ml) into a developing tank.
- Immediately cover the tank with the glass lid.
- Gently rock the tank to mix the solvents.
- Allow the tank to equilibrate for 15 minutes before use.

### STANDARDS

Carprofen

### PROCEDURE

#### Base Hydrolysis with Acid Extract (BH)

1. Add 6.0 ml urine to a 16 x 125 mm screw top tube.
2. Make entry into "Reagent Addition" log book.
3. Add 1.0 ml 1 N NaOH. Vortex until homogeneous. Incubate in 65°C water bath for 20 minutes. Cool tube.
4. Make entry into "Reagent Addition" log book.
5. Add 4.0 ml pH 2.0 buffer and 4.0 ml DCM.
6. Cap tube and rotorack 15 minutes. Centrifuge for 5 minutes.
7. Make entry into "Sample Handling" log book.
8. Aspirate aqueous (upper) layer and transfer organic layer to a clean 15 x 85 mm tube.
9. Concentrate to dryness in 60°C water bath.

10. Spot a portion of the residue on each of three TLC plates using DCM. The amount spotted is determined by the nature of the residue and through experience. Overspotting should be avoided. Thorough rinsing of spotting tip between samples is imperative.

#### **Plate #1 - Davidow**

1. Spot carprofen as standard.
2. Develop in Davidow (use 40-50 ml/tank) for 6 cm. Dry plate.
3. Observe with 365 nm UV light. Indicate fluorescence with =.
4. Observe with 254 nm UV light. Indicate quenching with ||.
5. Spray lightly with 4:4:2.
6. Spray with Dragendorff's. Record colors and Rf.
7. Spray with cupric chloride. Record colors and Rf. Allow plate to dry slightly.
8. Spray with sodium nitrite several times (wait between sprays). Record colors and Rf.

#### **Plate #2 - 4:4:2**

1. Spot carprofen as standard.
2. Develop in 4:4:2 chloroform/cyclohexane/acetic acid (use 40-50 ml/tank) for 4 cm.
3. Observe while wet with 365 nm UV light. Indicate fluorescence with =. Dry plate.
4. Observe with 365 nm UV light. Indicate fluorescence with =.
5. Observe with 254 nm UV light. Indicate quenching with ||.
6. Spray with Dragendorff's. Record colors and Rf.
7. Spray several times with sodium nitrite (wait between sprays for color change to develop). Record colors and Rf.
8. Spray with cupric chloride. Wait for 3 minutes. Record colors and Rf.

#### **Plate #3 - 9:1**

1. Spot carprofen as standard.
2. Develop for 4 cm in 9:1 chloroform/ethanol (use 40-50 ml/tank). Dry plate.
3. Observe with 365 nm UV light. Indicate fluorescence with =.
4. Observe with 254 nm UV light. Indicate quenching with ||
5. Spray with 4:4:2 and observe with 365 nm UV light. Indicate fluorescence with =.  
Dry plate.
6. Spray with Mandelin's. Record colors and Rf.
7. Place plate on medium hot plate for 3 minutes. Record colors and Rf. Cool plate.
8. Observe with 365 nm UV light. Note and record fluorescence. Observe **BOTH** sides of the plate.

#### **Notes on Procedure**

1. The time between steps 3 and 5 should be minimized, i.e. as soon as the tubes are cool the pH 2.0 buffer and DCM should be added.

2. When spraying the 9:1 plate with Mandelin's, meclofenamic acid appears as a jet black arch but quickly fades and can go undetected. Therefore, examine the plate immediately after application of the Mandelin's spray.

## DERIVITIZATION PROCEDURE

1. Add 20  $\mu$ l of TMAH and 20  $\mu$ l of ethyl acetate to the residue in each tube including the standard.
2. Cap and vortex mix the contents of each tube for 5-10 seconds.
3. Heat for 5 minutes at  $65 \pm 5^{\circ}\text{C}$ .
4. Remove the tubes from the heating block and submit for GC/MS analysis.

## GAS CHROMATOGRAPHIC AND MASS SPECTROMETER OPERATING PARAMETERS

- Instrumentation:  
Hewlett-Packard GC/MSD equipped with HP MS Chemstation operating software (MS-DOS and MS-Windows)
- GC Column:  
type: HP-5 or HP-1 (Hewlett-Packard)  
Length: 30 meters  
I.d.: 0.25 mm  
Film thickness: 0.25  $\mu\text{m}$
- Carrier Gas:  
type: Helium ultra-high purity (99.999%)  
flow rate: 1.0 ml/min  
column head pressure: 15 psi
- Injection  
type: 0.8 minute splitless  
injection volume: 1  $\mu$ l
- Autosampler  
type: model 7673 (Hewlett Packard)  
sample washes: 0  
sample pumps: 4  
viscosity delay: 1 second  
solvent washes: 4
- Temperatures:  
injector: 260 $^{\circ}\text{C}$   
oven temperature program: 80 $^{\circ}\text{C}$  (5.0 minute) increasing at 20 $^{\circ}\text{C}/\text{minute}$  to a final temperature of 275 $^{\circ}\text{C}$  (hold 13.74 minute)  
Interface: 280 $^{\circ}\text{C}$
- Source:  
pressure: 5-8 x 10 $^{-6}$  Torr  
temperature: determined by the interface
- Ionization:  
electron-impact

- Programs:
  - POSITIVE.M - full scan acquisition program
  - start time: 15.0 minutes
  - Low mass: 50
  - High mass: 550
  - Threshold: 10-5971, 1000-5970
  - EMV offset: 200

## Procedure

1. Transfer the contents to 100 µl autosampler vials using disposable pipets.
2. Order of analysis:
  - a) DFTPP instrument must pass tuning requirement
  - b) Standard the standard or standards for the drug or drugs being confirmed.
  - c) Blank a reagent blank of the same matrix as the sample, standards, and controls.
  - d) CNTL(-) a blank urine extracted the same way and at the same time as the sample(s).
  - e) Sample extract of urine containing suspect(s).
  - f) Blank a reagent blank of the same matrix as the samples, standards and controls.
  - g) CNTL(+) a blank urine which has been spiked with the drug(s) which is being confirmed, or a urine which has been previously confirmed to have that drug(s) present.
  - h) Blank a reagent blank of the same matrix as the samples standards and controls.
  - i) Standard the standard or standards for the drug or drugs being analyzed.
3. Acquire data for the calibrators using the CARPROFEN.M program only. Acquire data for the remaining sample extracts using both CARPROFEN.M and POSITIVE.M programs.

## Evaluation of Mass Spectral Data for Carprofen

1. For each sample extract, obtain the total ion chromatogram (TIC), the integrated ion area and retention time for the following monitored ions:  $m/z$  207, 242 and 301 (carprofen-dimethylated).
2. Extracted ion profiles of two or more major ions in the retention time area for each drug identified. This must be done for every injection pertinent to confirmation. Blanks used specifically for injector cleanup and the DFTPP injection are not included.
3. Mass spectral plots of the drug(s) under analysis. This includes the standards, sample(s), and the control positive(s).
4. Mass lists of each of the mass spectral plots mentioned in (2) above.
5. Library match information for each drug in each positive sample.

6. A mass spectral plot of DFTPP and a 1% mass list of this plot for the Mass Spectrometer.
7. GC/MS data review check sheet.

### **Criteria for Identification of Carprofen from Urine Extracts**

1. The retention times of the ions at  $m/z$  207, 242 and 301 amu from the test sample must be within  $\pm 1.0\%$  of the retention time of the same ions from a carprofen calibrator or standard.
2. The peak area ration for ions at  $m/z$  207, 242 and 301 amu, plus three additional ions, from the test sample must be within  $\pm 30\%$  of the values of the same ions from a carprofen calibrator or standard.
3. The full scan spectra of the test sample and the standard must have the same fragmentation pattern and retention time ( $\pm 1.0\%$ ).

### **RESPONSIBLE PERSONS**

- Analysts assigned to the Confirmation Section
- Supervisor of the Confirmation Section