

# **DEZOCINE: DETECTION AND CONFIRMATION**

Developed By

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**For Testing Integrity Program**

## **STANDARD OPERATING PROCEDURE FOR:**

### **ENZYME HYDROLYSIS EXTRACTION PROCEDURE FOR DEZOCINE**

#### **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.

Enzyme hydrolysis with a basic extract is used to liberate numerous drugs and drug metabolites of many types. This method is critical in the detection of narcotics, drugs such as isoxsuprine, methocarbamol and metabolites of many antihistamines, local anesthetics, alkaloids and tranquilizers.

#### **MATERIALS**

1. Science, silica gel, Fluorescent, Thin Layer Chromatography Plates
2. solid phase columns, Worldwide Monitoring™ Clean Screen Extraction
3. 100- $\mu$ l autosampler vials and caps
4. Capillary plate spotter
5. Hot plate with small fan
6. 16 x 125 mm screw top test tubes with caps
7. Vortex mixer
8. Automatic pipettor with 10 mL graduated pipettes
9. Rotorack mixer for test tubes
10. Centrifuge
11. Disposable Pasteur pipettes
12. Vacuum aspiration apparatus
13. Disposable 15 x 85 mm tubes
14. Light box with 254 nm and 365 nm U.V. light
15. Water bath
16. Hot Plate

## REAGENTS

### WATER

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

### 1 M SODIUM ACETATE BUFFER (PH 5.0)

#### Reagents

- sodium acetate, anhydrous, reagent grade
- glacial acetic acid, reagent grade
- water

#### Procedure

- dissolve 246 g of sodium acetate in 1000 ml of water
- add 99 ml glacial acetic acid
- dilute to 300 ml with water. Mix
- **Store at approximately 4°C**

### $\beta$ -GLUCURONIDASE REAGENT (2500 UNITS/ML IN 1 M ACETATE BUFFER, PH 5)

#### Reagents

- $\beta$ -glucuronidase from *Patella vulgata* (Sigma No. G-8132)
- 1 M sodium acetate buffer (pH 5.0)

#### Procedure

- Mix two vials containing 1 million units each of  $\beta$ -glucuronidase from *Patella vulgata* with sufficient acetate buffer to produce 800 ml of the mixture

### DILUTED AMMONIUM HYDROXIDE SOLUTION (1:1; V/V)

*Prepare solution fresh daily.*

*Prepare under a fume hood.*

#### Reagents

- concentrated ammonium hydroxide, reagent grade
- water

#### Procedure

- add an equal volume of concentrated ammonium hydroxide to an equal volume of water Mix

### 10% AQUEOUS ASCORBIC ACID SOLUTION

#### Reagents

- ascorbic acid, reagent grade

- water

Procedure

- dissolve 25 g of ascorbic acid into sufficient water to produce 250 ml of solution
- store at approximately 4°C

### **DICHLOROMETHANE - ISOPROPANOL (3:1; v/v)**

Reagents

- dichloromethane, spectrophotometric grade
- isopropanol, reagent grade

Procedure

- combine 2400 ml of DCM and 800 ml of isopropanol

### **6 N HYDROCHLORIC ACID SOLUTION**

*Prepare under a fume hood*

Reagents

- concentrated hydrochloric acid, reagent grade
- water

Procedure

- add an equal volume of concentrated HCl to an equal volume of water. Mix

**Dichloromethane**, reagent grade

**Methanol**, reagent grade

### **1 N POTASSIUM HYDROXIDE SOLUTION**

*Prepare under a fume hood*

Reagents

- potassium hydroxide pellets, reagent grade
- water

Procedure

- Dissolve 56.1 grams of potassium hydroxide pellets in approximately 900 ml of water.
- Dilute to 100 ml with water. Mix.

### **0.1M ACETATE BUFFER (PH 4.0)**

Reagents

- glacial acetic acid, reagent grade
- 1 N potassium hydroxide solution

- water

Procedure

- Mix 570  $\mu$ l of glacial acetic acid with 1.6 ml of 1 N potassium hydroxide solution.
- Dilute to 100 ml with water and adjust to pH 4 with 1 N potassium hydroxide solution or glacial acetic acid.

**DICHLOROMETHANE-ISOPROPANOL (80:20; v/v) WITH 2% AMMONIUM HYDROXIDE (ELUTION SOLVENT)**

*Prepare fresh daily*

*Prepare under a fume hood*

Reagents

- dichloromethane, reagent grade
- isopropanol, reagent grade
- concentrated ammonium hydroxide, reagent grade

Procedure

- Combine 80 ml of dichloromethane with 20 ml of isopropanol
- Add 2 ml of ammonium hydroxide. Mix.

**0.1 M POTASSIUM PHOSPHATE BUFFER (PH 6.0)**

Reagents

- monobasic potassium phosphate, reagent grade
- water
- 1 N potassium hydroxide solution

Procedure

- Dissolve 13.6 grams of monobasic potassium phosphate in 900 ml of water.
- Adjust the solution to pH 6.0 with 10 N potassium hydroxide solution.
- Dilute to 100 ml with water. Mix.
- **Store at approximately 4°C.**

**N, O-BIS (TRIMETHYLSILYL) TRIFLUOROACETIMIDE (BSTFA), PIERCE No. 38828**

**ETHYL ACETATE, REAGENT GRADE**

**AMMONIUM HYDROXIDE/WATER**

Reagents

- concentrated ammonium hydroxide

- water

#### Procedure

- Combine 500 ml of concentrated ammonium hydroxide and 500 ml water. Mix.

### 1.0 N SULFURIC ACID SOLUTION

*Prepare under a fume hood*

*Always add acid to water*

#### Reagents

- concentrated sulfuric acid
- water

#### Procedure

- Dilute 111 ml of concentrated sulfuric acid to 4000 ml with water. Mix.

### PROPIONIC ACID SOLVENT

#### Reagents

- chloroform
- methanol
- propionic acid

#### Procedure

- Combine 72 ml chloroform, 18 ml of methanol and 10 ml of propionic acid using a 100 ml graduated glass cylinder.
- Pour the solvent (50 ml) into a glass development 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.
- **Tanks should be fresh (not more than 2 hours after preparation)**

### 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.

### WORKING STANDARD SOLUTIONS

Dezocine standards (10 ng/μl in methanol or 100 ng/μl in methanol).

### PROCEDURE: ENZYME HYDROLYSIS (SEH)

1. Add 5.0 mL urine to three separate 16 x 125 mm screw top tubes.
2. To each tube add 2.0 mL pH 5 acetate buffer and 1.0 mL *Patella vulgata* enzyme solution. Vortex until homogeneous. Incubate in 65°C water bath for 3 hours. Cool
3. To each tube add 0.5 mL 10% ascorbic acid. Adjust pH to 9.0 with 1:1 NH<sub>4</sub>OH:H<sub>2</sub>O. This requires approximately 0.75 mL.
4. To each tube add 5.0 mL 10:1 DCM:IPA. Cap tube and rotorack for 5 minutes. Centrifuge for 5 minutes.
5. Aspirate aqueous (upper) layer and transfer organic layer to a clean 16 x 125 mm screw top tube.
6. To each tube add 3.0 mL 1.0N H<sub>2</sub>SO<sub>4</sub>. Cap tube and rotorack for 5 minutes. Centrifuge for 5 minutes.
7. Transfer acid aqueous (upper) layer to a clean 16 x 125 mm screw top tube with a disposable pipette.
8. Add 0.5 mL 10% ascorbic acid. Adjust pH to 9.0 with 1:1 NH<sub>4</sub>OH:H<sub>2</sub>O. This requires approximately 0.75 mL.
9. To each tube add 5.0 mL 10:1 DCM:IPA. Cap tube and rotorack for 5 minutes. Centrifuge for 5 minutes.
10. Aspirate aqueous (upper) layer and transfer organic layer to a clean 15 x 85 mm tube. Concentrate to dryness in 60°C water bath.
11. Spot the entire residue equally on TLC plates using 9:1 DCM:MeOH. Cool plates prior to development.
12. Develop the plate in Propionic Acid solvent to five (5) cm.
13. Observe using 365 nm U.V. light. Indicate fluorescence with =.
14. Observe using 254 nm U.V. light. Indicate quenching with ||.
15. Dry plate thoroughly. Do not allow the plate to become exposed to excess heat. Spray plate lightly with Dragendroff's. Record colors and Rf's.

## **CRITERIA FOR IDENTIFICATION OF DEZOCINE BY THIN-LAYER CHROMATOGRAPHY**

Dezocine is identified by comparison of the color reactions and the R<sub>f</sub> values obtained from the extract of the test sample and the dezocine standard. Color reactions must be the same and the R<sub>f</sub> values must agree within  $\pm 0.05$  R<sub>f</sub> units.

## **SAMPLE PREPARATION FOR SOLID PHASE EXTRACTION**

1. Sonicate 20mls of urine with a horn sonicator at maximum intensity setting for at least 1.5 minutes. Use pulse mode; 1 or 2 second pulses. Turn intensity up slowly over the first 10 to 20 seconds.
2. Enzyme Hydrolysis of Glucuronide:  
To 20 ml of urine add 4 ml of  $\beta$  - glucuronidase and 8 mls of 1 M acetate buffer (pH 5.0). Mix/vortex. Hydrolyze for 3 hours at 65°C, or incubate over night at room temp. Cool before proceeding. Adjust sample pH to  $6.0 \pm 0.5$  using 6 N hydrochloric acid or diluted ammonium hydroxide solution.

## **SOLID PHASE EXTRACTION**

1. Place stopcocks on the air evacuation ports of the solid phase apparatus. Rinse each of these stopcock/needle assemblies with water and then methanol.
2. Rinse each of the stopcocks with the elution solvent, dichloromethane-isopropanol (80:20; v/v) with 2% ammonium hydroxide solution.
3. Place a conical tube labeled as the solvent blank under the collection needle designated for the test sample and pass 2 ml of the elution solvent through the stopcock into the conical tube.
4. Remove the solvent blank tube and then place the solid phase columns on the stopcocks.
5. Rinse the solid phase columns with 2 ml of methanol.
6. Rinse the solid phase columns with 2 ml of water; turn the stopcocks off before the liquid reaches the sorbent bed of the column
7. Pour each extract into its designated column and turn the stopcocks to the on position. Allow the solutions to drip slowly through the column.
8. When the extracts have passed through the columns, rinse columns with 2 ml of H<sub>2</sub>O.
9. Rinse each column with 1 ml of 0.1 M acetate buffer, pH 4.0.
10. Rinse each column with 2 ml of methanol; verify that all of the methanol has been drawn through the solid phase column with vacuum.
11. Turn off the vacuum, wipe the tips of the needles on the vacuum chamber with Kimwipe®, and place each labeled conical tube under its respective needle assembly.
12. Elute with 2 ml of 2% ammonium hydroxide solution in dichloromethane:isopropanol (80:20; v/v).
13. Evaporate the organic layer to dryness at  $65 \pm 5^\circ\text{C}$  under nitrogen.

## DERIVITIZATION PROCEDURE

1. Add 20  $\mu$ l of BSTFA 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 20 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:  $5-8 \times 10^{-6}$  Torr  
temperature: determined by the interface
- Ionization:  
electron-impact
- Programs:  
POSITIVE.M - full scan acquisition program

start time: 10.0 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 pipettes.
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 DEZOCINE.M program only. Acquire data for the remaining sample extracts using both DEZOCINE.M and POSITIVE.M programs.

## EVALUATION OF MASS SPECTRAL DATA FOR DEZOCINE

1. For each sample extract, obtain the total ion chromatogram (TIC), the integrated ion area and retention time for the following monitored ions:  $m/z$  128, 300 and 389 (dezocine diTMS) and 56, 243 and 300 (dezocine monoTMS).
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 DEZOCINE FROM URINE EXTRACTS**

1. The retention times of the ions at  $m/z$  128, 300 and 389 amu from the test sample must be within  $\pm 1.0\%$  of the retention time of the same ions from a dezocine calibrator or standard.
2. The peak area ration for ions at  $m/z$  128, 300 and 389 amu, plus three additional ions, from the test sample must be within  $\pm 30\%$  of the values of the same ions from a dezocine 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