

**IDENTIFICATION AND DETERMINATION OF BENZYLPIPERAZINE FROM HORSE URINE  
SAMPLES**

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**IDENTIFICATION AND DETERMINATION OF BENZYLPIPERAZINE  
FROM HORSE URINE SAMPLES**

**PREPARED BY**

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**FOR**

**TESTING INTEGRITY PROGRAMS, INC.  
P.O. BOX 18993  
Philadelphia, PA 19119 USA**

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**I. INTRODUCTION**

Benzylpiperazine (Figure 1) is a substance that was recently reported from official test samples collected from horses in several racing jurisdictions. Benzylpiperazine has reportedly been marketed as a product for treatment of respiratory ailments in horses although no published scientific studies on its effects in horses have been published. The substance is reportedly a central nervous system stimulant similar to amphetamine and methamphetamine (Bye et al., 1973). It is not a Drug Enforcement Administration controlled substance and has not been classified by the Association of Racing Commissioners International, Inc.

**II. SCOPE**

This standard operating procedure specifies procedures to be used for the identification and quantitation of benzylpiperazine from horse urine. The lower limit of quantitation for this method is approximately 10 ng/mL of urine.

**III. PRINCIPLE OF METHOD**

This standard operating procedure is to be used for the identification and quantitation of benzylpiperazine (Figure 1) from horse urine.

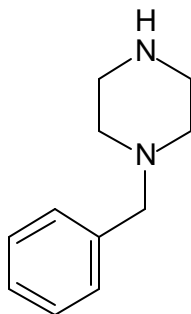


Figure 1. Benzylpiperazine.

In this procedure, benzylpiperazine is isolated from urine by solid phase extraction. The extract is evaporated to dryness, the residue is dissolved in aqueous ammonium hydroxide, and the resulting solution is re-extracted in order to remove unrelated components. Pentafluoropropionyl acid anhydride is used to produce the pentafluoropropionyl (PFP) amide derivative of benzylpiperazine. The derivative is identified and determined by gas chromatography / mass spectrometry. The concentrations of the analytes are determined by the internal standard method using peak area and linear regression analysis of calibration standards. Pseudoephedrine-d<sub>3</sub> is used as the internal standard.

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**IV. REAGENTS**

- A. Water, use Barnstead Nanopure water or equivalent in any reagent or procedure requiring the use of water.
  
- B. 1 N Hydrochloric acid solution
  - 1. Reagents
    - a) Concentrated hydrochloric acid, reagent grade
    - b) Water
  - 2. Procedure
    - a) Prepare under a fume hood.
    - b) Add 42 mL of concentrated hydrochloric acid to sufficient water to produce 500 mL of solution. Mix.
  - 3. Storage Requirements
    - a) Store at room temperature in a glass container.
    - b) Discard 1 year after preparation.
  
- C. 0.1 M Acetic Acid
  - 1. Reagents
    - a) Glacial acetic acid, reagent grade
    - b) Water
  - 2. Procedure
    - a) Prepare under a fume hood.
    - b) Add 2.86 mL of glacial acetic acid to 40 mL of water. Dilute to 50 mL and mix.
  - 3. Storage Requirements
    - a) Store at room temperature in a glass or plastic container.
    - b) Discard 6 months after preparation.
  
- D. 0.1 M Sodium Phosphate Buffer (pH 6.0)
  - 1. Reagents
    - a) Monobasic sodium phosphate, reagent grade
    - b) Dibasic sodium phosphate, reagent grade

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- c) Water
- 2. Procedure
  - a) Dissolve 1.70g of  $\text{Na}_2\text{HPO}_4$  and 12.10g  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  in 500 mL water. Mix.
  - b) Dilute to 1000 mL with water. Mix.
  - c) If necessary, adjust the pH by dropwise addition of 2 N NaOH or 1 N HCl
- 3. Storage Requirements
  - a) Store at approximately 4 °C in a glass container.
  - b) Discard 1 year after preparation.
- E. Elution Solvent
  - 1. Reagents
    - a) Dichloromethane, (cat. no. AH300-4, B&J, Muskegon, MI 49442-6184 or equivalent)
    - b) Isopropanol, reagent grade
    - c) Concentrated ammonium hydroxide, reagent grade
  - 2. Procedure
    - a) Prepare under a fume hood.
    - b) Combine 2 mL of ammonium hydroxide and 20 mL of isopropanol, Mix.
    - c) Add 78 mL of dichloromethane. Mix.
  - 3. Storage Requirements
    - a) Prepare the reagent fresh daily.
    - b) Store at room temperature in a glass container.
- F. Ethyl Acetate (Fisher Scientific, Optima Grade or equivalent)
- G. Methanol (Fisher Scientific, Optima Grade or equivalent)
- H. 2 N Sodium Hydroxide Solution (Fisher Scientific, Certified Grade or equivalent)
- I. Pentafluoropropionyl acid anhydride (PFPA, cat. no. 65193, Pierce, Rockford, IL 61101)
- J. Nitrogen gas.

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**V. SUPPLIES**

- A. 16 x 125 mm glass culture tubes with screw caps
- B. 12 x 75 mm glass culture tubes with caps
- C. Laboratory film (Parafilm® "M", American National Can, Chicago, IL, or equivalent).
- D. 2.0-mL autosampler vials with 250- $\mu$ L glass flange inserts, and 11-mm aluminum seals with PTFE rubber septa (cat. no. 24386, 24516, 21175, Restek Corporation, Bellefonte, PA 16823, or equivalent).
- E. Glass pasteur pipettes, disposable.
- F. Solid Phase Extraction Columns, CleanScreen® DAU, 3 mL, 130 mg (cat. no. CSDAU133, United Chemical Technologies, Bristol, PA 19007).

**VI. APPARATUS**

- A. Pipettes and tips  
**Note: Use the following positive displacement pipettes for pipetting the standard solutions and working standard solutions.**
  - 1. 0.1 - 10  $\mu$ L adjustable volume pipette (Eppendorf 2100, Brinkmann Instruments Inc., Westbury, NY 11590-0207).
  - 2. 2.0 - 20  $\mu$ L adjustable volume pipette (Eppendorf 2000, Brinkmann Instruments Inc., Westbury, NY 11590-0207).
  - 3. 10 - 100  $\mu$ L adjustable volume pipette (Eppendorf 2000, Brinkmann Instruments Inc., Westbury, NY 11590-0207).
  - 4. 20 - 200  $\mu$ L adjustable volume pipette (Eppendorf 2000, Brinkmann Instruments Inc., Westbury, NY 11590-0207).
  - 5. 100 - 1000  $\mu$ L adjustable volume pipette (Eppendorf 2000, Brinkmann Instruments Inc., Westbury, NY 11590-0207).
  - 6. 500 - 5000  $\mu$ L adjustable volume pipette (Eppendorf 2100, Brinkmann Instruments Inc., Westbury, NY 11590-0207).
- B. Vortex mixer (Glas-Col® Apparatus Co. Terre Haute, IN 47802 or equivalent)

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- C. pH meter (Corning 445, Fisher Scientific Co., Pittsburgh, PA 15219 or equivalent)
- D. Bransonic Ultrasonic Water Bath, 5510 (Fisher Scientific Co., Pittsburgh PA 15219 or equivalent)
- E. Centrifuge (Sorvall Super T21, Kendro Laboratory Products, Newtown, CT 06470 or equivalent)
- F. Rotorack (Glas-Col® Apparatus Co., Terre Haute, IN 47802 or equivalent).
- G. Cerex 24 or 48-place solid phase extraction apparatus (Cera Inc. Baldwin Park, CA 91706)
- H. Evaporator (TurboVap, Zymark, Cambridge, MA or equivalent).
- I. Heating block (Techne Instruments Inc or equivalent).

**VII. TEST SUBSTANCE**

Horse urine

**VIII. VOLUME REQUIRED**

1 mL or appropriate dilution. Refer to section IX.D for instructions regarding dilutions of the test samples.

**IX. STANDARD SOLUTIONS**

- A. Benzylpiperazine standard solution – 1.0 mg/mL

Prepare a 1.0 mg/mL Benzylpiperazine standard solution from the Benzylpiperazine reference standard and then two 10.00 ng/μL Benzylpiperazine working solutions. Use one working solution for the preparation of the calibrators and the standard and the other Benzylpiperazine working solution for the preparation of the positive control samples.

- 1. Reagents
  - a) 1-Benzylpiperazine standard (cat. no. 13683-2, Sigma Chemical Co., St. Louis, MO)
  - b) Methanol

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2. Procedure
  - a) Dissolve in methanol to prepare a 1.0-mg/mL stock standard solution as per SOP B001.
  - b) Store the standard solution at approximately -14 °C.
  
- B. d-Pseudoephedrine-d<sub>3</sub> standard solution – nominally 100 µg/mL (cat. no. FSD-082-100, Cambridge Isotope Laboratories, Andover, MA, 01810 or equivalent).

**X. WORKING STANDARD SOLUTIONS**

- A. Benzylpiperazine working standard solution in methanol - 10.0 ng/µL
  1. Reagents
    - a) Benzylpiperazine standard solution – 1.0 mg/mL
    - b) Methanol
  
  2. Procedure
    - a) Dilute 100 µL of the 1.0-mg/mL standard solution of benzylpiperazine to 10.0 mL with methanol.
    - b) Store the working standard solution at approximately -14 °C
  
- B. Benzylpiperazine working standard solution in methanol - 1.0 ng/µL
  1. Reagents
    - a) Benzylpiperazine working standard solution in methanol - 10.0 ng/µL
    - b) Methanol
  
  2. Procedure
    - a) Dilute 1.0 mL of the 10.0 ng/µL benzylpiperazine working standard solution in methanol to 10.0 mL with methanol.
    - b) Store the working standard solution at approximately -14 °C
  
- C. d-Pseudoephedrine-d<sub>3</sub> working standard solution in methanol - 10.0 ng/µL
  1. Reagents
    - a) d-Pseudoephedrine-d<sub>3</sub> standard solution - 100 µg/mL (cat. no. FSD-082-100, Cambridge Isotope Laboratories, Andover, MA, 01810 or equivalent).
    - b) Methanol
  
  2. Procedure

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- a) Dilute 200  $\mu\text{L}$  of d-pseudoephedrine- $\text{d}_3$  standard solution to 2.0 mL with methanol.
- b) Store the standard solution and working standard solution at approximately  $-14^\circ\text{C}$  when not in use.

**XI. CONTROL SAMPLES**

- A. Negative control horse urine - Horse urine sample negative for benzylpiperazine.
- B. Benzylpiperazine positive control urine 100 ng/mL - Add 10  $\mu\text{L}$  of 10.0 ng/ $\mu\text{L}$  benzylpiperazine working standard solution and 10  $\mu\text{L}$  of pseudoephedrine- $\text{d}_3$ - (see Table 1) working standard solution to each of three tubes, evaporate the methanol under nitrogen, and dilute to 1.0 mL with negative control horse urine.
- C. Include an administration positive control urine sample - if available.

**XII. SAMPLE REQUIREMENTS FOR ANALYSIS**

The following samples and standards are required for each analysis:

1. Calibrators designated **C<sub>1</sub>**, **C<sub>2</sub>**, **C<sub>3</sub>**, **C<sub>4</sub>**, **C<sub>5</sub>**, **C<sub>6</sub>** and **C<sub>7</sub>**; prepare calibrators at concentrations of 10, 25, 50, 100, 200, 300, and 400 ng/mL from negative control horse urine and the benzylpiperazine working standard solution.
2. System Blanks designated **SYS<sub>1</sub>** and **SYS<sub>2</sub>**; prepare system blanks from ethyl acetate.
3. Negative control sample designated **NC**.
4. Test sample(s) designated **TS<sub>1a...TS<sub>nb</sub></sub>** where n is the total number of test samples; a and b are designations for sample replicates.
5. Solvent blank(s) designated **SB<sub>1a...SB<sub>nb</sub></sub>** where n is the total number of test samples; a and b are designations for sample replicates.
6. Positive control samples designated **PC<sub>a</sub>**, **PC<sub>b</sub>**, and **PC<sub>c</sub>**; a, b, and c are designations for sample replicates.
7. Standard mixture designated **S<sub>1</sub>**.

**XIII. CALIBRATOR AND SAMPLE PREPARATION**

- A. Pipette 10  $\mu\text{L}$  of pseudoephedrine- $\text{d}_3$  working standard solution into each labeled 16 x 100 mm test tube except those labeled **SYS<sub>1</sub>**, **SYS<sub>2</sub>**, **SB<sub>1a...SB<sub>nb</sub></sub>** and **S<sub>1</sub>**.

**NOTE:** Prepare **S<sub>1</sub>** and **SYS<sub>1</sub>** and **SYS<sub>2</sub>** during steps XI.K and XI.L, respectively.

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- B. Pipette 10, 25, and 50  $\mu\text{L}$  of the 1.0-ng/ $\mu\text{L}$  benzylpiperazine working standard solution into the calibrator tubes labeled **C<sub>1</sub>**, **C<sub>2</sub>**, and **C<sub>3</sub>**. Pipette 10, 20, 30, and 40  $\mu\text{L}$  of the 10-ng/ $\mu\text{L}$  benzylpiperazine working standard solution into the calibrator tubes labeled **C<sub>4</sub>**, **C<sub>5</sub>**, **C<sub>6</sub>**, and **C<sub>7</sub>**, respectively. See Table 1. Evaporate the methanol under nitrogen before adding the urine.
- C. Pipette 10  $\mu\text{L}$  of benzylpiperazine working standard solution No.2 into the positive control tubes labeled PC<sub>a</sub>, PC<sub>b</sub>, PC<sub>c</sub>, respectively. Evaporate the methanol under nitrogen. See Table 1.

Table 1. Volumes of working standard solutions required to prepare calibrators, control samples, and test samples.

TUBE NO.	Volume of benzylpiperazine Working Standard Solution, $\mu\text{L}$	Volume of pseudoephedrine-d <sub>3</sub> Working Standard Solution, $\mu\text{L}$	Benzylpiperazine Injected into GC/MS (ng)	Pseudoephedrine-d <sub>3</sub> Injected into GC/MS (ng)	Equivalent to benzylpiperazine in the Urine (ng/mL)	Equivalent to pseudoephedrine-d <sub>3</sub> in the Urine (ng/mL)
C <sub>1</sub>	10 <sup>1</sup>	10	0.20	2.00	10	100
C <sub>2</sub>	25 <sup>1</sup>	10	0.80	2.00	25	100
C <sub>3</sub>	50 <sup>1</sup>	10	1.60	2.00	50	100
C <sub>4</sub>	10 <sup>2</sup>	10	2.40	2.00	100	100
C <sub>5</sub>	20 <sup>2</sup>	10	4.00	2.00	200	100
C <sub>6</sub>	30 <sup>2</sup>	10	6.00	2.00	300	100
C <sub>7</sub>	40 <sup>2</sup>	10	8.00	2.00	400	100
NC	0	10	0	2.00	0	100
TS <sub>1a-1b</sub>	0	10	unknown	2.00	unknown	100
SB <sub>1a-1b</sub>	0	0	0	0	na	na
PC <sub>a-c</sub>	10	10	2.0	2.00	100	100
S <sub>1</sub>	20	10	4.0	2.00	na	na

na = not applicable

<sup>1</sup> 1.0-ng/mL benzylpiperazine working standard solution

<sup>2</sup> 10-ng/mL benzylpiperazine working standard solution

- D. Pipette 1.0 mL of negative control urine into the tubes labeled **NC**, **PCa-c**, **C<sub>1</sub>**, **C<sub>2</sub>**, **C<sub>3</sub>**, **C<sub>4</sub>**, **C<sub>5</sub>**, **C<sub>6</sub>**, and **C<sub>7</sub>**.
- E. Pipette 1.0 mL of the test sample, in duplicate, into the tubes labeled **TS<sub>1a</sub>** and **TS<sub>1b</sub>**, if the estimated benzylpiperazine concentration in the test sample is between 10 and 400 ng/mL. If the estimated concentration is greater than 400 ng/mL, prepare an appropriate dilution of an aliquot of the test sample with negative control urine and pipette 1.0 mL of the diluted sample into the tubes labeled **TS<sub>1a</sub>** and **TS<sub>1b</sub>**. Repeat this process for each test sample.
- F. Pipette 1 mL of water into each of the tubes labeled **SB<sub>1a</sub>**..**SB<sub>nb</sub>**.

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- G. Vortex mix the contents of each tube for 5-10 seconds.

**XIV. SAMPLE PREPARATION FOR SOLID PHASE EXTRACTION**

- A. Dilute the contents of each tube with 1.6 mL of pH 6.0, 0.1 M phosphate buffer. If necessary, adjust the contents of each tube to pH  $6 \pm 0.5$  by dropwise addition of 2 N NaOH or 1 N HCl
- B. Centrifuge for 5 - 10 minutes at 2000-3000 rpm to remove sediment, if necessary.

**XV. SOLID PHASE EXTRACTION PROCEDURE**

- A. Wipe off the gasket with methanol soaked Kimwipe, then a dry Kimwipe.
- B. Place the labeled solid phase columns in the rack and condition each solid phase column by applying a small amount of pressure (maximum flow rate of 1 - 2 mL/minute) and successively eluting to waste 3 mL of methanol and 3 mL of 0.1 M phosphate buffer, pH 6.0. Ensure that all of the methanol has entered the sorbent bed before adding the buffer to prevent the methanol from mixing with the buffer and causing precipitation of buffer salts.
- C. Decant the contents of each tube into the corresponding column reservoir and adjust the flows so that the solutions flow slowly through the columns (*i.e.*, maximum flow rate of 1 - 2 mL/minute).
- D. Rinse each column with 3 mL of water.
- E. Rinse each column with 2 mL of 1 M acetic acid.
- F. Rinse each column with 3 mL of methanol.
- G. Dry the columns with high pressure N<sub>2</sub> flow for 2 minute.
- H. Place labeled 12 x 75 mm tubes into position under the corresponding cartridges. Verify that the tips are positioned into the tubes.
- I. Elute to collect with 2 mL of the elution solvent (78:20:2 CH<sub>2</sub>Cl<sub>2</sub>:iPrOH:NH<sub>4</sub>OH) all of the samples. Allow to soak 1 – 2 minutes after solvent enters cartridge. Verify that all the elution solvent has passed through the columns and turn low pressure nitrogen on (4-5 psi) for 10 - 20 seconds to expel residual solvent (Little or no pressure should be needed for elution).
- J. Evaporate the extracts of each tube to dryness at  $30 \pm 5$  °C under nitrogen.  
**IMPORTANT:** Watch the tubes carefully and remove each tube as soon as the extract is completely evaporated.
- K. Prepare the standard mixture by adding 20 µL of 10-ng/µL benzylpiperazine working standard solution and 10 µL of pseudoephedrine- d3 working standard solution to a 12 x 75 mm conical tube labeled **S<sub>1</sub>**.
- L. Prepare the system blank tubes by labeling two 12 x 75 mm conical tubes **SYS<sub>1</sub>** and **SYS<sub>2</sub>**.

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**XVI. DERIVATIZATION**

- A. Prepare the system wash tubes by labeling two 12 x 75 mm conical tubes **SYS<sub>1</sub>** and **SYS<sub>2</sub>**.
- B. Add 80 µL of pentafluoropropionyl acid anhydride (PFPA).
- C. Cap, parafilm tightly (stench) and vortex mix the contents of each tube for 20 - 30 seconds.
- D. Place the tubes in a heating block at 65 ± 5 °C for 30 minutes.
- E. Remove the tubes and evaporate PFPA in turbovap at 40°C.
- F. Add 80 µL of ethyl acetate, vortex mix and transfer to a labeled autosampler vial with insert.
- G. Submit for GC/MS analysis.

**XVII. GAS CHROMATOGRAPHIC/MASS SPECTRAL IDENTIFICATION OF BENZYLPIPERAZINE**

- A. Gas Chromatographic and Mass Spectrometer Operating Parameters
  - 1. Instrumentation:
    - a) Hewlett-Packard GC/MSD equipped with HP MS Chemstation operating software (MS-Windows NT)
  - 2. GC column:
    - a) column type: DB-5MS (J&W Scientific)
    - b) column length: 30 meters
    - c) column i.d.: 0.25 mm
    - d) film thickness: 0.25 µm
  - 3. Carrier gas:
    - a) type: Helium ultra-high purity (99.999%)
    - b) flow rate: 1.0 mL/min
    - c) column head press: 18.52 psi
  - 4. Injection:
    - a) type: pulsed splitless (40 psi until 0.5 min)
    - b) injection volume: 1 µL

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5. Autosampler:
  - a) type: model 7673 (Hewlett-Packard)
  - b) sample washes: 0
  - c) sample pumps: 3
  - d) viscosity delay: 1 seconds
  - e) solvent washes: 6
  
6. Temperatures:
  - a) injector: 250 °C
  - b) oven temp. program: 90 °C increase at 20 °C/minute to 250° and then 35°/min. to 300° C (hold for 7 minutes and return to 90 °C)
  - c) interface: 280 °C
  
7. Source:
  - a) pressure: 5 - 8 x 10<sup>-6</sup> Torr
  - b) temperature: determined by the interface
  
8. Ionization: electron-impact
  
9. Programs:
  - a) Name: BENZYLSI.M - selected ion monitoring program
  - b) Start time: 4 minutes
  - c) Dwell time: 25 msec
  - d) EMV offset: +200 V
  - e) Ions: Group 1 (start 4.0 min.): 119, 163, 207, and 297 amu
  - f) Ions: Group 2 (start ~6 min.): 91, 146, 175, 231, 245, and 322 amu
  - g) Name: BENZYLFS.M - Full scan program
  - h) Start time: 4 minutes
  - i) Low mass: 50
  - j) High mass: 500
  - k) Threshold: 150

**B. Procedure**

1. Transfer the contents of each tube to an autosampler vial using a new pipette for each transfer. Perform analysis in the order indicated in Table 2:

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**Table 2.** Run number, vial number, acquisition method, and sample designation for GC / MS analysis for identification and determination of benzylpiperazine from horse urine.

Run #	Vial	Method	Sample
1-7	1-7	BENZYLSI.M	C <sub>1</sub> , C <sub>2</sub> , C <sub>3</sub> , C <sub>4</sub> , C <sub>5</sub> , C <sub>6</sub> , C <sub>7</sub>
8-9	8-9	BENZYLSI.M	SYS <sub>1</sub> , SYS <sub>2</sub>
10	10	BENZYLSI.M	NC
11	10	BENZYLFS.M	NC
12	11	BENZYLSI.M	TS <sub>1a</sub>
13	11	BENZYLFS.M	TS <sub>1a</sub>
14	12	BENZYLSI.M	SB <sub>1a</sub>
15	12	BENZYLFS.M	SB <sub>1a</sub>
16	13	BENZYLSI.M	TS <sub>1b</sub>
17	13	BENZYLFS.M	TS <sub>1b</sub>
18	14	BENZYLSI.M	SB <sub>1b</sub>
19*	14	BENZYLFS.M	SB <sub>1b</sub>
20-22	15-17	BENZYLSI.M	PC <sub>a</sub> , PC <sub>b</sub> , and PC <sub>c</sub>
23	18	BENZYLSI.M	S <sub>1</sub>
24	19	BENZYLFS.M	S <sub>1</sub>

\*Analyze additional test sample extracts and solvent blanks by duplicating the specified sequence indicated in runs 12-19.

**C. Evaluation of Mass Spectral Data For Benzylpiperazine**

- For each test sample, calibrator, and positive control sample, obtain the total ion chromatogram (TIC), the integrated ion areas (A<sub>175</sub>, A<sub>231</sub>, A<sub>245</sub>, and A<sub>322</sub> and retention times for the benzylpiperazine derivative the integrated ion areas (A<sub>163</sub> and A<sub>207</sub>) and retention times for the for d3-pseudoephedrine.
- Calculate the ion area ratios by dividing the ion areas of the following ions: m/z 175 and 231 (245 may be used as alternate ion) by the ion area of the ion at m/z 322 for benzylpiperazine for each replicate of the test sample and the standard.
- Measure the signal-to-noise ratio for both replicates of each test sample for the peak for the ion at m/z 175 (or 245-if used) at the retention time of the peak for benzylpiperazine.

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4. At the retention times of benzylpiperazine, print the full scan spectra from the negative control sample extract, test sample extracts, standard and solvent blank from the corresponding data files.
- D. Criteria for Identification of Benzylpiperazine from Urine Extracts
1. The retention times of the ions at m/z 175, 231, and 322 (or 245\*)amu for each replicate of the test sample must be within  $\pm 0.05$  minutes of the retention time of the same ions from the benzylpiperazine peak in the standard.
  2. The relative ion area ratio for ions at m/z 175, 231, and 322 (or 245) amu from each replicate of the test sample must be within  $\pm 20\%$  of the values of the same ions from the standard.
  3. The chromatographic peak shape must be approximately Gaussian, with a narrow base, with baseline separation from neighboring peaks, and with little evidence of tailing. The following criteria will define an acceptable peak:
    - a) The width of the peak at its base should be less than 0.20 minutes.
    - b) The peak should appear to be Gaussian, i.e, symmetrical about the vertical mid-line.
    - c) There should be no interfering peaks. A neighboring peak is considered to be interfering if the height from the baseline to the lowest part of the valley between the peaks is greater than 20% of the height of the peak of interest.
    - d) There is no significant peak tailing. Unacceptable peak tailing is defined as the condition in which the ratio of b to a is greater than 1.5 at 15% of the peak height.
  4. The full scan spectra of benzylpiperazine from the test sample and the standard have the same fragmentation pattern and the retention time is within 0.05 minutes.
- E. Determination of the Concentration of Benzylpiperazine in Horse Urine
1. Plot the peak area ratio of m/z 322 at the retention time of benzylpiperazine and m/z 207 at the retention time of pseudoephedrine-d<sub>3</sub>-IS for each calibrator versus the concentration of benzylpiperazine in the calibrator. Perform linear regression analysis on these data to obtain the slope, intercept, and correlation coefficient of the standard curve.

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\* Ion 245 may be used as an alternate ion if coeluting materials interfere with one ion current.

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2. Calculate the concentration of benzylpiperazine in each test sample or diluted test sample and positive control sample from the peak area ratios of m/z 322/207 and the slopes and intercepts of the standard curves.
3. Calculate the concentration of benzylpiperazine in each diluted test sample from the calculated concentration and the dilution factor used:

$$\text{Concentration} = \text{Calculated concentration} / \text{dilution factor}$$

4. Determine the mean concentration for each test sample and positive control sample.

$$\text{Mean concentration} = \frac{1}{2} (\text{concentration TS}_{1a} + \text{concentration TS}_{1b})$$

**XVIII. CRITERIA FOR REPEATING THE ANALYSIS**

Repeat the analysis of the test sample if any of the following conditions apply:

- A. The peak area ratio of any test sample replicate is greater than the peak area ratio of highest calibrator. Repeat the analysis after diluting the urine sample with water as described in Section IX.D of this Standard Operating Procedure.
- B. The negative control sample or the solvent blanks contain benzylpiperazine as evidenced by the presence of the characteristic ions within the expected retention time window.
- C. The standard curve for benzylpiperazine has a correlation coefficient less than 0.98.
- D. The pseudoephedrine-d<sub>3</sub> ions are not detectable within the expected retention time window for any of the sample replicates.
- E. The average concentration of the positive control sample replicates is not within 25% of the nominal concentration.

**XIX. CRITERIA FOR REPORTING A POSITIVE SAMPLE**

- A. Report a test sample as positive when all of the following criteria are met:
  1. The mean benzylpiperazine concentration in the test sample is greater than 10 ng/mL.
  2. The signal-to-noise ratio of the ion at m/z 175 (or 245-if used as alternate ion) for the benzylpiperazine peak in each replicate of the test sample is greater than 3.
  3. The negative control sample and the system and solvent blanks do not contain benzylpiperazine.

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4. Mean benzylpiperazine concentration in the positive controls is within 25% of the nominal value.
5. The standard curves have correlation coefficients greater than 0.98.

**XX. REFERENCES**

- A. Varian Sample Preparation Products. Bond Elut Certify Solid Phase Extraction Application Manual, Harbor City, CA, February, 1995.
- B. Kamerling S.G. And Barker S.A., (1994) Stimulants: Effects on Behaviour and Performance, Proceedings of the 10<sup>th</sup> International Conference of Racing Analysts and Veterinarians.
- C. Sams, R. A. (1999) The Ohio State Analytical Toxicology Laboratory, Standard Operating Procedure for Identification and Determination of Amphetamine. 858: 1-20.
- D. Bye C., Munro Faure A.D., Peck A.W., and Young P.A. (1973) A comparison of the effects of 1-benzylpiperazine and dexamphetamine on performance tests. Comparison of the effects of dexamphetamine and 1-benzylpiperazine in former addicts. *European Journal of Clinical Pharmacology* **6**: 163-169.