

## IDENTIFICATION AND DETERMINATION OF NEFOPAM IN HORSE PLASMA BY LIQUID CHROMATOGRAPHY/MASS SPECTROMETRY

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### I. INTRODUCTION

Nefopam (Figure 1) is a centrally-acting, non-narcotic analgesic drug that is used to treat moderate to severe pain caused by injury, surgery, and cancer. Nefopam also has anticholinergic and sympathomimetic effects. It is marketed in the United States as oral tablets and a parenteral formulation (20 mg/mL) as Acupan<sup>®</sup>.

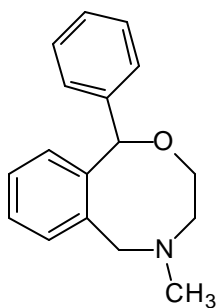


Figure 1 Chemical structure of nefopam.

Nefopam is classified as a class 3 drug by the Association of Racing Commissioners International and the Ohio State Racing Commission due to its potential to affect performance of a horse in competition.

### II. SCOPE OF METHOD

This standard operating procedure specifies procedures to be used to identify and determine nefopam from extracts of horse plasma using liquid chromatography/mass spectrometry. This procedure is to be used after nefopam has been identified in the corresponding urine sample using the identification procedure found in Section 834 of the OSU Analytical Toxicology Laboratory Standard Operating Procedures Manual "IDENTIFICATION AND DETERMINATION OF NEFOPAM IN HORSE URINE BY GAS CHROMATOGRAPHY/MASS SPECTROMETRY".

### III. PRINCIPLE OF METHOD

Nefopam and the internal standard, phencyclidine-*d*<sub>5</sub>, are isolated from buffered plasma using solid-phase extraction. The analytes are eluted from the extraction column using dichloromethane/isopropanol/ammonium hydroxide. The eluates are dried, dissolved in aqueous formic acid, and analyzed by liquid chromatography/mass spectrometry.

IDENTIFICATION AND DETERMINATION OF NEFOPAM IN HORSE PLASMA BY LIQUID  
CHROMATOGRAPHY/MASS SPECTROMETRY

---

operated under electrospray ionization conditions in the positive ion MS/MS mode of operation. The concentration of nefopam in the sample is determined by the internal standard method using peak area ratios and linear regression analysis. The lower limit of quantitation for this method has not been determined. The method is linear in the range of 100 to 4000 pg/mL.

IV. REAGENTS

A. Water

1. Unless otherwise specified, use water that meets requirements for Type II reagent water as defined by the National Committee for Clinical Laboratory Standards (Preparation and testing of reagent water in the clinical laboratory - third edition; approved guideline C3-A3. Wayne, PA: NCCLS, 1997).
2. Use water suitable for HPLC applications for the preparation of mobile phase components (processed water from Barnstead Nanopure™ Infinity Deionization System, Barnstead International, Dubuque, Iowa 52001-1478, or cat. no. 365-4, B&J, Muskegon, MI 49442-6184, or equivalent).

B. 0.1 N Acetic acid solution

1. Reagents
  - a. Glacial acetic acid, ACS reagent grade
  - b. Water
2. Procedure
  - a. **Caution: Prepare under a fume hood.**
  - b. Add 1.5 mL of glacial acetic acid to sufficient water to produce 250 mL of solution. Mix.
3. Storage Requirements
  - a. Store at room temperature in a glass container.
  - b. Discard 1 year after preparation.

C. Saturated sodium borate reagent

IDENTIFICATION AND DETERMINATION OF NEFOPAM IN HORSE PLASMA BY LIQUID  
CHROMATOGRAPHY/MASS SPECTROMETRY

---

1. Reagents
    - a. Sodium tetraborate decahydrate, granular, ACS reagent grade
    - b. Water
  2. Procedure
    - a. Add 500 g of sodium tetraborate to 3500 mL of water. Mix, observing for saturation by incomplete dissolution of reagent.
    - b. Add more sodium tetraborate, if needed, to ensure saturation as indicated by the presence of non-dissolved solid.
  3. Storage Requirements
    - a. Store at room temperature in a glass container.
    - b. Discard 1 year after preparation.
- D. Elution Solvent
1. Reagents
    - a. Dichloromethane, ACS reagent grade or better (cat. no. 300-4, B&J, or equivalent)
    - b. Isopropanol, ACS reagent grade or better (cat. no. 3032-08, Mallinckrodt Baker, Paris, KY 40361, or equivalent)
    - c. Concentrated ammonium hydroxide, ACS reagent grade
  2. Procedure
    - a. **Caution: Prepare under a fume hood.**
    - b. Combine 2 mL of ammonium hydroxide and 20 mL of isopropanol. Mix.
    - c. Slowly add 78 mL of dichloromethane while swirling.
  3. Storage Requirements
    - a. Prepare the reagent fresh daily.
    - b. Store at room temperature in a glass container.
- E. Acetonitrile, ACS reagent grade or better, suitable for HPLC applications (cat. no. 015-4, B&J, or equivalent)

IDENTIFICATION AND DETERMINATION OF NEFOPAM IN HORSE PLASMA BY LIQUID  
CHROMATOGRAPHY/MASS SPECTROMETRY

---

- F. Formic acid, 88%, ACS reagent grade or better (cat. no. A118P-500, Fisher Scientific, Fair Lawn, NJ 07410, or equivalent)
- G. 0.1% Aqueous formic acid solution (HPLC mobile phase component "A")
1. Reagents
    - a. Formic acid
    - b. Water
  2. Procedure
    - a. **CAUTION: Prepare under a fume hood.**
    - b. Add 1.0 mL of formic acid to approximately 900 mL of water and dilute to 1000 mL of solution. Mix.
    - c. Outgas by placing the container in an ultrasonic bath for approximately 2 minutes.
  3. Storage Requirements
    - a. Store at approximately 4 °C in a glass container.
    - b. Discard six months after preparation.
- H. 0.1% Formic acid solution in acetonitrile (HPLC mobile phase component "B")
1. Reagents
    - a. Formic acid
    - b. Acetonitrile
  2. Procedure
    - a. **CAUTION: Prepare under a fume hood.**
    - b. Add 1.0 mL of formic acid to approximately 900 mL of acetonitrile and dilute to 1000 mL of solution. Mix.
    - c. Outgas by placing the container in an ultrasonic bath for approximately 2 minutes.
  3. Storage Requirements

IDENTIFICATION AND DETERMINATION OF NEFOPAM IN HORSE PLASMA BY LIQUID  
CHROMATOGRAPHY/MASS SPECTROMETRY

---

- a. Store at room temperature in a glass container.
- b. Discard six months after preparation.

I. Methanol, ACS reagent grade or better (cat. no. 230-4, B&J, or equivalent)

J. Nitrogen gas

V. **SUPPLIES**

A. 16 x 125-mm glass culture tubes

B. 13 x 100-mm glass conical centrifuge tubes with caps

C. Solid phase extraction columns, 3-mL, 30 mg, sorbent type DAU (cat. no. 532-DAU, ANSYS Technologies, Inc., Lake Forest, CA 92630)

D. 2-mL glass autosampler vials (cat. no. C4011-2W, National Scientific, Duluth, GA 30097, or equivalent), low-volume glass inserts (cat. no. C4010-627L, National Scientific, or equivalent) and 11-mm aluminum seals with PTFE/silicone rubber septa (cat. no. C4011-4A, National Scientific, or cat. no. 24359, Restek Corporation, Bellefonte, PA 16823, or equivalent). NOTE: Do not use PTFE/red rubber septa, e.g., Restek cat. no. 21175.

E. Tissue paper wipers (e.g., Kimwipes<sup>®</sup>)

F. Glass pasteur pipettes, disposable

VI. **APPARATUS**

A. Pipettes

**Note: Use positive displacement pipettes for pipetting all standard solutions. Plasma specimens may be pipetted using either positive displacement or air displacement pipettes.**

1. 1 - 10- $\mu$ L positive displacement pipette (microman cat. no. m10, Rainin Instrument Co., Inc., Woburn, MA 01888-4026, or equivalent).

2. 10 - 100- $\mu$ L positive displacement pipette (microman cat. no. m100, Rainin Instrument Co., Inc., or equivalent).

**IDENTIFICATION AND DETERMINATION OF NEFOPAM IN HORSE PLASMA BY LIQUID  
CHROMATOGRAPHY/MASS SPECTROMETRY**

---

3. 200 - 1000- $\mu$ L positive displacement pipette (microman cat. no. m1000, Rainin Instrument Co., Inc., or equivalent).
  4. 10 - 100- $\mu$ L adjustable volume pipettor (Eppendorf 4810, Brinkmann Instruments Inc., Westbury, NY 11590, or equivalent).
  5. 100 - 1000- $\mu$ L adjustable volume pipettor (pipet-plus, cat. no. R-1000, Rainin Instrument Co., Inc., or Eppendorf 4810, Brinkmann Instruments Inc., or equivalent).
  6. 1 - 10-mL electronic pipettor (edp plus, Rainin Instrument Co., Inc.) or 2 - 10-mL adjustable volume pipette (Finnpipette<sup>®</sup>, Fisher Scientific, Pittsburgh, PA 15275), or equivalent.
  7. 2.5-mL gastight<sup>®</sup> blunt tip syringe and repetitive dispenser (syringe model no. 81416, dispenser model no. PB600-1, Hamilton Co., Reno, NV 89502, or equivalent).
- B. Vortex mixer (American Scientific Products, McGaw Park, IL 60085, or equivalent).
- C. Ultrasonic bath capable of sonicating mobile phase containers and 13 x 100-mm glass conical centrifuge tubes (Bransonic model 220, Branson Instruments Co., Shelton, CT, 06484, or equivalent).
- D. Centrifuge capable of centrifuging 13 x 100-mm glass conical tubes at 2000 - 3000 rpm (Damon/IEC division, model HN-S, Needham Heights, MA 02194, or equivalent).
- E. Solid phase extraction manifold (Varian Vac Elut SPS 24<sup>™</sup> cat. no. 1223-4022, Varian Sample Preparation Products, Harbor City, CA 90710, or equivalent).
- F. Evaporator capable of evaporating solvent to dryness from 13 x 100-mm glass conical tubes at  $40 \pm 5$  °C under nitrogen (The Meyer N-Evap, Organomation Assoc. Inc., South Berlin, MA 01549, or equivalent).

**VII. TEST SUBSTANCE**

The test substance specified in this procedure is horse plasma.

**IDENTIFICATION AND DETERMINATION OF NEFOPAM IN HORSE PLASMA BY LIQUID CHROMATOGRAPHY/MASS SPECTROMETRY**

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**VIII. VOLUME REQUIRED**

Analyze duplicate 1.0-mL aliquots of undiluted test sample (2 mL total) if the estimated concentration of nefopam in the test sample is between 100 and 4000 pg/mL. If the estimated concentration of nefopam is greater than 4000 pg/mL, prepare a dilution of an aliquot of the test sample with negative control plasma so that the estimated concentration of nefopam in the diluted sample is between 100 and 4000 pg/mL.

**IX. GRAVIMETRIC AND REFERENCE STANDARD SOLUTIONS**

**A. Nefopam Gravimetric Standard Solution "A" –1.0 mg/mL**

1. Reagents
  - a. Nefopam hydrochloride reference standard – (cat. no. 01372, Alltech-Applied Science, State College, PA 16801)
  - b. Methanol
2. Procedure
  - a. Quantitatively transfer 11.4 mg of nefopam hydrochloride reference standard to a 10-mL volumetric flask.
  - b. Dissolve in and dilute to volume with methanol. Mix.
3. Storage Requirements
  - a. Store the standard solution at 2 - 8 °C and protected from light.
  - b. Discard the standard solution 1 month after preparation.

**B. Nefopam Gravimetric Standard Solution "B" –1.0 mg/mL**

1. Reagents
  - a. Nefopam hydrochloride reference standard – (cat. no. 01372, Alltech-Applied Science)
  - b. Methanol
2. Procedure

**IDENTIFICATION AND DETERMINATION OF NEFOPAM IN HORSE PLASMA BY LIQUID  
CHROMATOGRAPHY/MASS SPECTROMETRY**

---

- a. Quantitatively transfer 11.4 mg of nefopam hydrochloride reference standard to a 10-mL volumetric flask.
  - b. Dissolve in and dilute to volume with methanol. Mix.
3. Storage Requirements
    - a. Store the standard solution at 2 - 8 °C and protected from light.
    - b. Discard the standard solution 1 month after preparation.
- C. Phencyclidine- $d_5$  reference standard solution - nominally 100  $\mu\text{g}/\text{mL}$  in methanol (cat. no. P-003, Cerilliant Corp., Austin, TX 78708, or equivalent). Store the standard solution at 2 - 8 °C and protected from light.

**X. INTERMEDIATE AND WORKING STANDARD SOLUTIONS**

- A. Nefopam Intermediate Standard Solution "A"- 10 ng/ $\mu\text{L}$
1. Reagents
    - a. Nefopam gravimetric standard solution "A" (1.0 mg/mL)
    - b. Methanol
  2. Procedure
    - a. Pipette 100  $\mu\text{L}$  of nefopam gravimetric standard solution "A" (1.0 mg/mL) into a 10-mL volumetric flask.
    - b. Dilute to volume with methanol and mix.
  3. Store the intermediate standard solution at 2 - 8 °C and protected from light.
- B. Nefopam Working Standard Solution "A"- 100 pg/ $\mu\text{L}$
1. Reagents
    - a. Nefopam intermediate standard solution "A" (10 ng/ $\mu\text{L}$ )
    - b. Methanol
  2. Procedure

IDENTIFICATION AND DETERMINATION OF NEFOPAM IN HORSE PLASMA BY LIQUID  
CHROMATOGRAPHY/MASS SPECTROMETRY

---

- a. Pipette 100  $\mu\text{L}$  of nefopam intermediate standard solution "A" (10  $\text{ng}/\mu\text{L}$ ) into a 10-mL volumetric flask.
    - b. Dilute to volume with methanol and mix.
  3. Use nefopam working standard solution "A" (100  $\text{pg}/\mu\text{L}$ ) for the preparation of the standard, **S**<sub>1</sub>, and calibrators **C**<sub>5</sub>, **C**<sub>6</sub>, **C**<sub>7</sub>, **C**<sub>8</sub>, and **C**<sub>9</sub>.
  4. Store the working standard solution at 2 - 8 °C and protected from light.
- C. Nefopam Working Standard Solution "A"- 10  $\text{pg}/\mu\text{L}$ 
  1. Reagents
    - a. Nefopam working standard solution "A" (100  $\text{pg}/\mu\text{L}$ )
    - b. Methanol
  2. Procedure
    - a. Pipette 1.0 mL of nefopam working standard solution "A" (100  $\text{pg}/\mu\text{L}$ ) into a 10-mL volumetric flask.
    - b. Dilute to volume with methanol and mix.
  3. Use nefopam working standard solution "A" (10  $\text{pg}/\mu\text{L}$ ) for the preparation of calibrators **C**<sub>1</sub>, **C**<sub>2</sub>, **C**<sub>3</sub>, and **C**<sub>4</sub>.
  4. Store the working standard solution at 2 - 8 °C and protected from light.
- D. Nefopam Intermediate Standard Solution "B"- 10  $\text{ng}/\mu\text{L}$ 
  1. Reagents
    - a. Nefopam gravimetric standard solution "B" (1.0  $\text{mg}/\text{mL}$ )
    - b. Methanol
  2. Procedure
    - a. Pipette 100  $\mu\text{L}$  of nefopam gravimetric standard solution "B" (1.0  $\text{mg}/\text{mL}$ ) into a 10-mL volumetric flask.
    - b. Dilute to volume with methanol and mix.

IDENTIFICATION AND DETERMINATION OF NEFOPAM IN HORSE PLASMA BY LIQUID  
CHROMATOGRAPHY/MASS SPECTROMETRY

---

3. Store the intermediate standard solution at 2 - 8 °C and protected from light.
- E. Nefopam Intermediate Standard Solution “**B**”- 1.0 ng/μL
1. Reagents
    - a. Nefopam intermediate standard solution “**B**” (10 ng/μL)
    - b. Methanol
  2. Procedure
    - a. Pipette 1.0 mL of nefopam intermediate standard solution “**B**” (10 ng/μL) into a 10-mL volumetric flask.
    - b. Dilute to volume with methanol and mix.
  3. Store the intermediate standard solution at 2 - 8 °C and protected from light.
- F. Nefopam Working Standard Solution “**B**”- 10 pg/μL
1. Reagents
    - a. Nefopam intermediate standard solution “**B**” (1.0 ng/μL)
    - b. Methanol
  2. Procedure
    - a. Pipette 100 μL of nefopam intermediate standard solution “**B**” (1.0 ng/μL) into a 10-mL volumetric flask.
    - b. Dilute to volume with methanol and mix.
  3. Use nefopam working standard solution “**B**” (10 pg/μL) for the preparation of the positive control samples.
  4. Store the working standard solution at 2 - 8 °C and protected from light.
- G. Phencyclidine-*d*<sub>5</sub> Intermediate Internal Standard Solution - 1.0 ng/μL

IDENTIFICATION AND DETERMINATION OF NEFOPAM IN HORSE PLASMA BY LIQUID  
CHROMATOGRAPHY/MASS SPECTROMETRY

---

1. Reagents
  - a. Phencyclidine- $d_5$  reference standard solution (100  $\mu\text{g}/\text{mL}$ )
  - b. Methanol
2. Procedure
  - a. Pipette 100  $\mu\text{L}$  of phencyclidine- $d_5$  reference standard solution (100  $\mu\text{g}/\text{mL}$ ) into a 10-mL volumetric flask.
  - b. Dilute to volume with methanol and mix.
3. Store the intermediate internal standard solution at 2 - 8  $^{\circ}\text{C}$  and protected from light.

H. Phencyclidine- $d_5$  Working Internal Standard Solution – 200  $\text{pg}/\mu\text{L}$

1. Reagents
  - a. Phencyclidine- $d_5$  intermediate internal standard solution (1.0  $\text{ng}/\mu\text{L}$ )
  - b. Methanol
2. Procedure
  - a. Pipette 2.0 mL of phencyclidine- $d_5$  intermediate internal standard solution (1.0  $\text{ng}/\mu\text{L}$ ) into a 10-mL volumetric flask.
  - b. Dilute to volume with methanol and mix.
3. Store the working internal standard solution at 2 - 8  $^{\circ}\text{C}$  and protected from light.

XI. CONTROL SAMPLES

- A. Negative control plasma – Horse plasma sample demonstrated by analysis to contain no detectable nefopam. Store at approximately – 20  $^{\circ}\text{C}$ .
- B. Positive control plasma designated **PC<sub>1</sub>** – Negative control plasma supplemented with nefopam at 250  $\text{pg}/\text{mL}$ . Preparation of the positive control sample is described in section XIII.

IDENTIFICATION AND DETERMINATION OF NEFOPAM IN HORSE PLASMA BY LIQUID  
CHROMATOGRAPHY/MASS SPECTROMETRY

---

- C. Positive control plasma designated **PC<sub>2</sub>** – Negative control plasma supplemented with nefopam at 750 pg/mL. Preparation of the positive control sample is described in section XIII.

XII. **SAMPLE REQUIREMENTS FOR ANALYSIS**

Prepare the following samples and standards for each analysis:

- A. 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>, C<sub>7</sub>, C<sub>8</sub>, and C<sub>9</sub>**; prepare calibrators at concentrations of 100, 250, 500, 750, 1000, 1500, 2000, 3000, and 4000 pg/mL, respectively, from negative control horse plasma and nefopam working standard solutions "**A**" (10 pg/μL and 100 pg/μL).
- B. System washes designated **SYS<sub>1</sub>** and **SYS<sub>2</sub>**; prepare system washes from 0.1% aqueous formic acid solution.
- C. Negative control sample designated **NC**; prepare negative control sample from negative control horse plasma.
- D. Test sample designated **TS<sub>1a</sub>** and **TS<sub>1b</sub>**; **a** and **b** are designations for test sample replicates.
- E. Solvent blank designated **SB<sub>1a</sub>** and **SB<sub>1b</sub>**; **a** and **b** are designations for solvent blank replicates.
- F. Positive control samples designated **PC<sub>1a-1b</sub>** and **PC<sub>2a-2b</sub>**; **a** and **b** are designations for control sample replicates. Prepare positive control samples from negative control horse plasma and nefopam working standard solution "**B**" (10 pg/μL).
- G. Standard mixture designated **S<sub>1</sub>**.

XIII. **CALIBRATOR AND SAMPLE PREPARATION**

- A. Pipette 50 μL of phencyclidine-*d*<sub>5</sub> working internal standard solution (200 pg/μL) into each labeled 16 x 125-mm tube except those labeled **S<sub>1</sub>, SYS<sub>1</sub>, SYS<sub>2</sub>, SB<sub>1a</sub>, and SB<sub>1b</sub>**. See Table 1.

**NOTE:** Prepare **S<sub>1</sub>, SYS<sub>1</sub>** and **SYS<sub>2</sub>**, and **SB<sub>1a</sub>** and **SB<sub>1b</sub>** in steps XIV.P, XIV.S, and XIV.D, respectively.

**STANDARD OPERATING PROCEDURES  
OSU ANALYTICAL TOXICOLOGY LABORATORY**

Section Code: 615  
Effective Date: 12/04/02  
Supersedes: DRAFT  
Page: 13 of 24

**IDENTIFICATION AND DETERMINATION OF NEFOPAM IN HORSE PLASMA BY LIQUID CHROMATOGRAPHY/MASS SPECTROMETRY**

- B. Pipette 10, 25, 50, and 75  $\mu\text{L}$  of nefopam working standard solution “A” (10  $\text{pg}/\mu\text{L}$ ) into the calibrator tubes labeled **C<sub>1</sub>**, **C<sub>2</sub>**, **C<sub>3</sub>**, and **C<sub>4</sub>**, respectively. Pipette 10, 15, 20, 30, and 40  $\mu\text{L}$  of nefopam working standard solution “A” (100  $\text{pg}/\mu\text{L}$ ) into the calibrator tubes labeled **C<sub>5</sub>**, **C<sub>6</sub>**, **C<sub>7</sub>**, **C<sub>8</sub>**, and **C<sub>9</sub>**, respectively. See Table 1.

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

TUBE NO.	Volume of Nefopam Working Standard Solution, $\mu\text{L}$	Volume of PCP- <i>d</i> <sub>5</sub> Working Internal Standard Solution, $\mu\text{L}$	Nefopam Injected into LC/MS, pg	PCP- <i>d</i> <sub>5</sub> Injected into LC/MS, ng	Equivalent to Nefopam in the Plasma, $\text{pg}/\text{mL}$	Equivalent to PCP- <i>d</i> <sub>5</sub> in the Plasma, $\text{ng}/\text{mL}$
<b>C<sub>1</sub></b>	10 <sup>1</sup>	50	20	2	100	10
<b>C<sub>2</sub></b>	25 <sup>1</sup>	50	50	2	250	10
<b>C<sub>3</sub></b>	50 <sup>1</sup>	50	100	2	500	10
<b>C<sub>4</sub></b>	75 <sup>1</sup>	50	150	2	750	10
<b>C<sub>5</sub></b>	10 <sup>2</sup>	50	200	2	1000	10
<b>C<sub>6</sub></b>	15 <sup>2</sup>	50	300	2	1500	10
<b>C<sub>7</sub></b>	20 <sup>2</sup>	50	400	2	2000	10
<b>C<sub>8</sub></b>	30 <sup>2</sup>	50	600	2	3000	10
<b>C<sub>9</sub></b>	40 <sup>2</sup>	50	800	2	4000	10
<b>SYS<sub>1-2</sub></b>	0	0	0	0	na	na
<b>NC</b>	0	50	0	2	0	10
<b>TS<sub>1a-1b</sub></b>	0	50	unknown	2	unknown	10
<b>SB<sub>1a-1b</sub></b>	0	50	0	2	na	na
<b>PC<sub>1a-1b</sub></b>	25 <sup>3</sup>	50	50	2	250	10
<b>PC<sub>2a-2b</sub></b>	75 <sup>3</sup>	50	150	2	750	10
<b>S<sub>1</sub></b>	50 <sup>2</sup>	100	500	2	na	na

na = not applicable

<sup>1</sup> volume of nefopam working standard “A” 10  $\text{pg}/\mu\text{L}$

<sup>2</sup> volume of nefopam working standard “A” 100  $\text{pg}/\mu\text{L}$

<sup>3</sup> volume of nefopam working standard “B” 10  $\text{pg}/\mu\text{L}$

**IDENTIFICATION AND DETERMINATION OF NEFOPAM IN HORSE PLASMA BY LIQUID CHROMATOGRAPHY/MASS SPECTROMETRY**

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- C. Pipette 25  $\mu$ L of nefopam working standard solution “B” (10 pg/ $\mu$ L) into each of the tubes labeled **PC<sub>1a</sub>** and **PC<sub>1b</sub>**.
- D. Pipette 75  $\mu$ L of nefopam working standard solution “B” (10 pg/ $\mu$ L) into each of the tubes labeled **PC<sub>2a</sub>** and **PC<sub>2b</sub>**.
- E. Add 2.0 mL of saturated sodium borate solution to each test tube. Vortex-mix the contents of each tube for 3 - 5 seconds.
- F. Pipette 1.0 mL of negative control plasma into the tubes labeled **NC**, **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>**, **C<sub>8</sub>**, **C<sub>9</sub>**, **PC<sub>1a</sub>**, **PC<sub>1b</sub>**, **PC<sub>2a</sub>**, and **PC<sub>2b</sub>**.
- G. Pipette a 1.0-mL aliquot of the test sample into each of the tubes labeled **TS<sub>1a</sub>** and **TS<sub>1b</sub>** if the estimated concentration of nefopam in the test sample is between 100 and 4000 pg/mL. If the estimated concentration of nefopam is greater than 4000 pg/mL, prepare an appropriate dilution of an aliquot of the test sample with negative control plasma and pipette a 1.0-mL aliquot of the diluted sample into each of the tubes labeled **TS<sub>1a</sub>** and **TS<sub>1b</sub>**.
- H. Vortex-mix the contents of each tube for 3 - 5 seconds.

**XIV. SOLID PHASE EXTRACTION OF NEFOPAM**

- A. Place a stopcock for each test sample replicate, calibrator, and control sample replicate onto the stainless steel delivery tips. Plug the ports that are not in use with port sealing plugs.
- B. Rinse the stopcocks and needles by successively eluting to waste approximately 10 mL of water, 10 mL of methanol, and 2 mL of elution solvent.
- C. Remove the extraction manifold lid and wipe off the collection needles with tissue paper wipers.
- D. Place 13 x 100-mm conical tubes labeled **SB<sub>1a</sub>** and **SB<sub>1b</sub>** in the collection rack positions that will be used for the corresponding test sample replicates. Collect 1 mL of elution solvent. Remove the tubes and set them aside until step XIV.Q.
- E. Place a solid phase extraction column on each stopcock. Condition each column by applying a small amount of vacuum (1-5 mm Hg) and successively eluting to waste 1 mL of methanol and 1 mL of water. Stop the flow as soon as each reagent reaches the top of the sorbent bed.

IDENTIFICATION AND DETERMINATION OF NEFOPAM IN HORSE PLASMA BY LIQUID  
CHROMATOGRAPHY/MASS SPECTROMETRY

---

- F. Decant each prepared test sample replicate, calibrator, and control sample replicate into the corresponding column reservoirs and adjust the flows so that the solutions flow through the columns in not less than 1 minute.
- G. Increase the vacuum briefly to draw a small volume of air through each column.
- H. Rinse each column with 1 mL of water.
- I. Increase the vacuum briefly to draw a small volume of air through each column.
- J. Rinse each column with 1 mL of 0.1 *N* acetic acid.
- K. Dry the columns under full vacuum for approximately 2 minutes.
- L. Rinse each column with 1 mL of methanol.
- M. Dry the columns under full vacuum for approximately 1 minute.
- N. Place labeled conical tubes into position under the corresponding collection needles. Verify that the needles are positioned into the tubes.
- O. Elute to collect with 0.5 mL of the elution solvent. Increase the vacuum briefly to draw a small volume of air through each column. Repeat with a second 0.5-mL aliquot of elution solvent. Verify that all the elution solvent has been drawn through the columns, then turn the vacuum on full (15 – 20 inches Hg) for 5 - 10 seconds.
- P. Prepare the standard mixture, **S**<sub>1</sub>, by adding 50 μL of the nefopam working standard solution "**A**" (100 pg/μL) and 100 μL of phencyclidine-*d*<sub>5</sub> working internal standard solution (200 pg/μL) to a 13 x 100-mm conical tube labeled **S**<sub>1</sub>.
- Q. Pipette 50 μL of phencyclidine-*d*<sub>5</sub> working internal standard solution (200 pg/μL) into the solution contained in tubes **SB**<sub>1a</sub> and **SB**<sub>1b</sub>.
- R. Evaporate the contents of each tube to dryness under nitrogen in a water bath at 40 ± 5 °C.
- S. Prepare the system wash tubes by labeling two 13 x 100-mm conical test tubes **SYS**<sub>1</sub> and **SYS**<sub>2</sub>.

**IDENTIFICATION AND DETERMINATION OF NEFOPAM IN HORSE PLASMA BY LIQUID CHROMATOGRAPHY/MASS SPECTROMETRY**

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- T. Add 200  $\mu\text{L}$  of 0.1% aqueous formic acid to the tube labeled **S<sub>1</sub>**. Vortex-mix the contents for 5 - 10 seconds.
  - U. Add 100  $\mu\text{L}$  of 0.1% aqueous formic acid to each of the remaining tubes. Vortex-mix the contents for 5 – 10 seconds.
  - V. Cap and sonicate all tubes for approximately 6 minutes, then vortex-mix for 5 - 10 seconds.
  - W. Centrifuge the tubes for approximately 5 minutes at 2000 - 3000 rpm.
  - X. Carefully transfer the entire contents of each tube to a low-volume insert in an appropriately labeled autosampler vial, using a new disposable pipette for each transfer.
  - Y. Cap and submit the vials for LC/MS analysis.
- XV. LIQUID CHROMATOGRAPHIC/MASS SPECTRAL IDENTIFICATION OF NEFOPAM**
- A. Liquid Chromatograph and Mass Spectrometer Operating Parameters
    - 1. Instrumentation:

Agilent LC/MSD-Trap equipped with Agilent MS Chemstation operating software (MS-Windows)
    - 2. LC column:
      - a. Guard column: Metaguard™ Polaris™ C18-A (cat. no. 2000-MG2000, ANSYS Technologies, Inc.)
      - b. Column type: Metasil™ Polaris C18-A (cat. no. 2000-100X030, ANSYS Technologies, Inc.)
      - c. Column length: 100 mm
      - d. Column i.d.: 3.0 mm
      - e. Particle size: 5  $\mu\text{m}$
    - 3. Chromatography:
      - a. Program name: NEFOPLAS.M

**STANDARD OPERATING PROCEDURES  
OSU ANALYTICAL TOXICOLOGY LABORATORY**

Section Code: 615  
Effective Date: 12/04/02  
Supersedes: DRAFT  
Page: 17 of 24

**IDENTIFICATION AND DETERMINATION OF NEFOPAM IN HORSE PLASMA BY LIQUID  
CHROMATOGRAPHY/MASS SPECTROMETRY**

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- b. Mobile phase:
    - i. Component "A" 0.1% aqueous formic acid
    - ii. Component "B" 0.1% formic acid in acetonitrile
  - c. Flow rate: 0.3 mL/min
  - d. Gradient: 28% B isocratic
  - e. Column temperature: 35 °C
  - f. Stop time: 8 minutes
  - g. Injection volume: 20 µL
4. Ionization:
- a. Type: electrospray, positive ion
  - b. Resolution: normal
  - c. Nebulizer pressure: 40 psi
  - d. Dry gas temperature: 350 °C
  - e. Dry gas flow rate: 9 L/min
5. MS/MS program;
- a. Capillary voltage: -3500V
  - b. Solvent divert time: 3 minutes
  - c. Skimmer voltage: 45.0V
  - d. Capillary exit offset: 130.0V
  - e. Octapole 1 voltage: 6.00V
  - f. Octapole 2 voltage: 2.50V
  - g. Octapole RF voltage: 60.0Vpp
  - h. Trap drive: 45.0 (arbitrary units)
  - i. Lens 1 voltage: -5.0V
  - j. Lens 2 voltage: -60.0V
  - k. Maximum accumulation time: 100 msec
  - l. Scan range: 100-300 amu
  
  - m. Nefopam transition: 254.1 amu → scan
  - n. Nefopam isolation: 254.1 amu, isolation width 0.7 amu, fragmentation cutoff 105 amu, fragmentation amplitude 1.80
  
  - o. Nefopam ions: 165, 166, 179, and 181 amu
  - p. Scan averages: 3
  
  - q. PCP-*d*<sub>5</sub> transition: not applicable (MS only)
  - r. PCP-*d*<sub>5</sub> isolation: 249.1 amu, isolation width 1.2 amu
  - s. PCP-*d*<sub>5</sub> ion: 249.1 amu
  - t. Scan averages: 3

**IDENTIFICATION AND DETERMINATION OF NEFOPAM IN HORSE PLASMA BY LIQUID CHROMATOGRAPHY/MASS SPECTROMETRY**

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**B. Procedure**

Perform analyses in the order and with the acquisition program specified in Table 2:

**Table 2.** Order of analysis for identification and determination of nefopam in horse plasma samples.

Run #	Vial	Method	Sample
1-9	1-9	NEFOPLAS.M	<b>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>, C<sub>8</sub>, C<sub>9</sub></b>
10-11	10-11	NEFOPLAS.M	<b>SYS<sub>1</sub>, SYS<sub>2</sub></b>
12-13	12-13	NEFOPLAS.M	<b>PC<sub>1a</sub>, PC<sub>1b</sub></b>
14-15	14-15	NEFOPLAS.M	<b>PC<sub>2a</sub>, PC<sub>2b</sub></b>
16	16	NEFOPLAS.M	<b>NC</b>
17	17	NEFOPLAS.M	<b>TS<sub>1a</sub></b>
18	18	NEFOPLAS.M	<b>SB<sub>1a</sub></b>
19	19	NEFOPLAS.M	<b>TS<sub>1b</sub></b>
20	20	NEFOPLAS.M	<b>SB<sub>1b</sub></b>
21-22	12-13	NEFOPLAS.M	<b>PC<sub>1a</sub>, PC<sub>1b</sub></b>
23-24	14-15	NEFOPLAS.M	<b>PC<sub>2a</sub>, PC<sub>2b</sub></b>
25	21	NEFOPLAS.M	<b>S<sub>1</sub></b>

**C. Evaluation of Mass Spectral Data**

1. Obtain the total ion chromatogram (TIC) and determine the integrated ion areas ( $A_{ion(m/z)}$ ) and retention times for the qualifying ions for nefopam and phencyclidine- $d_5$  listed in Table 3 for each test sample extract, calibrator extract, positive control sample extract, and the standard.
2. Calculate the relative ion area ratios for nefopam by dividing each qualifying ion area by the ion area of the most abundant ion as indicated in Table 3 for each replicate of the test sample and the standard.

IDENTIFICATION AND DETERMINATION OF NEFOPAM IN HORSE PLASMA BY LIQUID  
CHROMATOGRAPHY/MASS SPECTROMETRY

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**Table 3.** Qualifying and quantifying ions for MS/MS analysis for nefopam in horse plasma samples; the most abundant ions are indicated in **bold** type and the least abundant qualifying ions are underlined.

Analyte	Qualifying Ions (amu)	Quantifying Ions (amu)
Nefopam	254 → <u>165</u> , 166, 179, <b>181</b> amu	<u>165</u> , 166, 179, <b>181</b> amu (summed)
Phencyclidine- <i>d</i> <sub>5</sub>	249 amu	249 amu

3. Calculate the peak area ratio for nefopam by dividing the area of the quantifying ion at the retention time of nefopam by the area of the corresponding quantifying ion at the retention time of phencyclidine-*d*<sub>5</sub> for each test sample replicate, calibrator, and positive control sample replicate.
  4. Measure the signal-to-noise ratio for the least abundant qualifying ion for nefopam in both replicates of the test sample. The least abundant qualifying ion is listed and underlined in Table 3.
  5. Print the MS/MS spectrum of nefopam from the standard full scan data file. Print the full scan spectra from the negative control sample extract, each test sample extract, and solvent blanks at the retention time of nefopam from the respective data files.
- D. Criteria for Identification of Nefopam in Plasma Sample Extracts
1. The retention times of the qualifying ions for nefopam in each test sample replicate must be within  $\pm 2.0\%$  of the retention times of the same ions for nefopam from the standard.
  2. The retention times of the qualifying ions for nefopam must be within one scan of each other.
  3. The retention time of the qualifying ion for phencyclidine-*d*<sub>5</sub> in each test sample replicate must be within  $\pm 2.0\%$  of the retention time of the same ion for phencyclidine-*d*<sub>5</sub> from the standard.

IDENTIFICATION AND DETERMINATION OF NEFOPAM IN HORSE PLASMA BY LIQUID  
CHROMATOGRAPHY/MASS SPECTROMETRY

---

4. The relative ion area ratios for the qualifying ions for nefopam in both replicates of the test sample must be within  $\pm 30\%$  of the ratios of the same ions from the standard. The most abundant ion must be the ion indicated in Table 3.
  5. 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 shape and width of the peak should be essentially the same as that of the standard.
    - 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 10% 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 where *a* is the time from the leading edge of the peak to the mid-line and *b* is the time from the mid-line to the trailing edge.
  6. The MS/MS spectrum of nefopam from each test sample extract and from the standard must have essentially the same fragmentation patterns.
- E. Determination of the Concentration of Nefopam in Horse Plasma
1. For each calibrator, plot the peak area ratios of the quantifying ion of nefopam versus the concentration of nefopam in the calibrator. Perform linear regression analysis on these data to obtain the slope, intercept, and correlation coefficient of the standard curve.
  2. Calculate the concentration of nefopam in each test sample replicate and control sample replicate from the peak area ratios of the quantifying ions and the slope and intercept of the standard curve. If the test sample has been diluted, apply the dilution factor to the calculation.

IDENTIFICATION AND DETERMINATION OF NEFOPAM IN HORSE PLASMA BY LIQUID  
CHROMATOGRAPHY/MASS SPECTROMETRY

---

Concentration = Calculated concentration/dilution factor

3. Determine the average concentration of nefopam in the test sample and each positive control sample.

Average concentration =  $\frac{1}{2}$  (concentration **TS**<sub>1a</sub> + concentration **TS**<sub>1b</sub>)

XVI. **CRITERIA FOR REPEATING THE ANALYSIS**

If any of the following conditions apply, investigate and correct the cause of the unacceptable result and repeat the analysis of the test sample:

- A. The negative control sample or the solvent blanks contain nefopam as evidenced by the presence of the characteristic ions within the expected retention time window.
- B. The standard curve for nefopam has a correlation coefficient less than 0.98.
- C. The ion for the internal standard is not detectable within the expected retention time window for either of the test sample replicates.
- D. The replicate nefopam concentrations measured in either **PC**<sub>1</sub> or **PC**<sub>2</sub> differ from the respective nominal concentrations by more than 30%.
- E. The replicate concentrations differ from the corresponding average concentrations by more than 15% for either **PC**<sub>1</sub> or **PC**<sub>2</sub>.
- F. The peak area ratio for nefopam in either test sample replicate is greater than the peak area ratio of the highest calibrator. Repeat the analysis after diluting the plasma sample as described in Section VIII of this standard operating procedure.
- G. The test sample replicate values differ from the average test sample value by more than 15%.
- H. There is an interfering substance in the test sample. Refer to Section XVIII.  
**INTERFERING SUBSTANCES.**

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

Report a test sample as positive for nefopam if all of the following criteria are met:

IDENTIFICATION AND DETERMINATION OF NEFOPAM IN HORSE PLASMA BY LIQUID  
CHROMATOGRAPHY/MASS SPECTROMETRY

---

- A. The test sample contains nefopam according to the identification criteria described in XV.D.
- B. The peak area ratio of nefopam in both test sample replicates is greater than the peak area ratio of the lowest calibrator.
- C. The signal-to-noise ratio of the least abundant qualifier ion in both replicates of the test sample is greater than 10.

XVIII. **INTERFERING SUBSTANCES**

No known substances have been found to interfere with the determination of nefopam by this procedure. It is possible that a large amount of one or more substances in the extract could inhibit the ionization of and therefore interfere with detection of nefopam or the internal standard. The latter would be evident by a reduction in the response of the internal standard. Appropriate corrective actions would include modification of the extraction procedure to reduce the amount of interfering substances, and modification of the chromatographic conditions to separate the interferences from nefopam or the internal standard.

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IDENTIFICATION AND DETERMINATION OF NEFOPAM IN HORSE PLASMA BY LIQUID CHROMATOGRAPHY/MASS SPECTROMETRY

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**STANDARD OPERATING PROCEDURES  
OSU ANALYTICAL TOXICOLOGY LABORATORY**

Section Code: 615  
Effective Date: 12/04/02  
Supersedes: DRAFT  
Page: 24 of 24

**IDENTIFICATION AND DETERMINATION OF NEFOPAM IN HORSE PLASMA BY LIQUID  
CHROMATOGRAPHY/MASS SPECTROMETRY**

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**XX. RESPONSIBLE PERSONNEL**

- A. Analysts assigned to the Confirmation Section
- B. Supervisor of the Confirmation Section

**Prepared by:**

Dr. Richard Sams, David Hall, Shelley Smith

**Approved by:**

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Lucille Kaminski - QAO                      Date

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Richard Sams - Director                      Date