

IDENTIFICATION OF TRAMADOL AND NEFOPAM IN HORSE URINE BY GAS CHROMATOGRAPHY/MASS SPECTROMETRY

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 several countries outside the United States as oral tablets and a parenteral formulation (20 mg/mL) as Acupan[®]. Nefopam is not approved for use in the United States in either human or veterinary medicine.

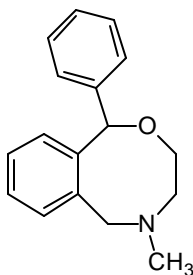


Figure 1 Chemical structure of nefopam.

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.

Tramadol (Figure 2) is a centrally-acting, analgesic drug that is used to treat moderate to moderately severe pain caused by injury, surgery, and cancer. It is marketed in the United States as oral tablets (50 mg) as Ultram[®]. Tramadol is metabolized primarily by O-demethylation to M1. Both tramadol and M1 bind to opioid receptors in the brain to produce analgesia.

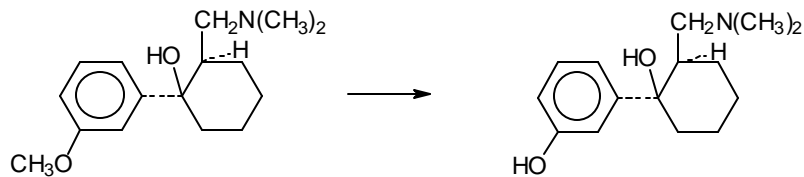


Figure 2 Chemical structures of tramadol and O-desmethyltramadol (M1).

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Tramadol is classified as a class 2 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 nefopam and tramadol from horse urine extracts using gas chromatography/mass spectrometry. This procedure may be used to determine if nefopam or tramadol is present in a test sample that produces a positive test result on the Tramadol ELISA test (Neogen, Inc.). Alternatively, this procedure may be used to screen samples for the presence of nefopam or tramadol. Procedures for confirmation and determination of nefopam in horse urine are described in the OSU Analytical Toxicology Laboratory *Standard Operating Procedures Manual Section 834 (Identification and Determination of Nefopam In Horse Urine By Gas Chromatography/Mass Spectrometry)*. Procedures for confirmation and determination of nefopam in horse plasma are described in the OSU Analytical Toxicology Laboratory *Standard Operating Procedures Manual Section 615 (Identification and Determination of Nefopam In Horse Plasma By Liquid Chromatography/Mass Spectrometry)*.

III. PRINCIPLE OF METHOD

Nefopam, tramadol, and the internal standard, phencyclidine-*d*₅, are extracted from basified horse urine by solid phase extraction. The eluates are dried, dissolved in BSTFA, and analyzed by gas chromatography/mass spectrometry operated under electron-impact ionization conditions in the selected ion monitoring mode. If either analyte is detected by this procedure, it is identified and determined by an analyte-specific procedure as described in the *OSU Analytical Toxicology Laboratory Standard Operating Procedures Manual*.

IV. REAGENTS

A. Water

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).

B. 0.1 N Acetic acid solution

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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. Elution Solvent
1. Reagents
 - a. Dichloromethane, ACS reagent grade or better (cat. no. 300-4, B&J, Muskegon, MI 49442-6184, 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. Mix.
 3. Storage Requirements
 - a. Prepare the reagent fresh daily.
 - b. Store at room temperature in a glass container.
- D. Saturated sodium borate reagent

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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.

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

F. *N,O*-bis-(trimethylsilyl)trifluoroacetamide (BSTFA; cat. no. 38830, Pierce Chemical Co., Rockford, IL 61101, or equivalent).

G. Nitrogen gas

V. SUPPLIES

A. 16 x 100-mm glass culture tubes.

B. 13 x 100-mm glass round-bottom culture tubes.

C. Solid phase extraction columns, 10-mL, 200 mg, sorbent type CSDAU (cat. no. ZSDAU020, United Chemical Technologies, Inc., Bristol, PA 19007).

D. 2-mL glass autosampler vials (cat. no. C4011-2W, National Scientific, Duluth, GA 30097, or equivalent), low-volume glass inserts (cat. no. C4010-629L, 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. Glass pasteur pipettes, disposable.

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- F. Tissue paper wipers (e.g., Kimwipes®).

VI. APPARATUS

- A. Pipettes

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

1. 1 - 10- μ L positive displacement pipette (microman cat. no. m10, Rainin Instrument Co., Inc., Woburn, MA 01888-4026, or equivalent).
 2. 10 - 100- μ L positive displacement pipette (microman cat. no. m100, Rainin Instrument Co., Inc., or equivalent).
 3. 200 - 1000- μ L positive displacement pipette (microman cat. no. m1000, Rainin Instrument Co., Inc., or equivalent).
 4. 10 - 100- μ L adjustable volume pipettor (Eppendorf 4810, Brinkmann Instruments Inc., Westbury, NY 11590, or equivalent).
 5. 100 - 1000- μ 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®, Fisher Scientific, Pittsburgh, PA 15275), or equivalent.
 7. 2.5-mL gastight® 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. Rotorack (Glas-Col® Apparatus Co., Terre Haute, IN 47802, or equivalent).

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- D. Centrifuge capable of centrifuging 16 x 100-mm glass culture tubes at 1500 - 2000 rpm (Damon/IEC division, model HN-S, Needham Heights, MA 02194, or equivalent).
- E. Ultrasonic bath capable of sonicating 13 x 100-mm round-bottom tubes (Bransonic model 220, Branson Instruments Co., Shelton, CT, 06484, or equivalent).
- F. Evaporator capable of evaporating solvent to dryness from 13 x 100-mm round-bottom tubes at 40 ± 5 °C under nitrogen (The Meyer N-Evap, Organomation Assoc. Inc., South Berlin, MA 01549, or equivalent).
- G. Solid phase extraction manifold (Varian Vac Elut SPS 24™ cat. no. 1223-4022, Varian Sample Preparation Products, Harbor City, CA 90710, or equivalent).

VII. TEST SUBSTANCE

The test substance specified in this procedure is horse urine.

VIII. VOLUME REQUIRED

Analyze a 1.0-mL aliquot of undiluted test sample.

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

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- a. Store the standard solution at 2 – 8 °C and protected from light.
 - b. Discard the standard solution 1 month after preparation.
- B. Tramadol hydrochloride reference standard solution – nominally 1.0 mg/mL of tramadol in methanol (cat. no. T-027, Cerilliant Corp., Austin, TX 78708, or equivalent). Store the standard solution at 2 - 8 °C and protected from light.
- C. Phencyclidine-*d*₅ reference standard solution – nominally 100 µg/mL in methanol (cat. no. P-003, Cerilliant Corp., or equivalent). Store the standard solution at 2 - 8 °C and protected from light.

X WORKING STANDARD SOLUTIONS

- A. Nefopam Working Standard Solution “**A**”- 10 ng/µL
- 1. Reagents
 - a. Nefopam gravimetric standard solution “**A**” (1.0 mg/mL)
 - b. Methanol
 - 2. Procedure
 - a. Pipette 100 µL of nefopam gravimetric standard solution “**A**” (1.0 mg/mL) into a 10-mL volumetric flask.
 - b. Dilute to volume with methanol and mix.
 - c. Store the working standard solution at 2 - 8 °C and protected from light.
- B. Tramadol Working Standard Solution - 10 ng/µL
- 1. Reagents
 - a. Tramadol reference standard solution – (1.0 mg/mL)
 - b. Methanol
 - 2. Procedure
 - a. Pipette 100 µL of tramadol reference standard solution (1.0 mg/mL) into a 10-mL volumetric flask.
 - b. Dilute to volume with methanol and mix.

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- c. Store the working standard solution at 2 - 8 °C and protected from light.
- C. Phencyclidine-*d*₅ Working Internal Standard Solution – 1.0 ng/μL
 1. Reagents
 - a. Phencyclidine-*d*₅ reference standard solution (100 μg/mL)
 - b. Methanol
 2. Procedure
 - a. Pipette 100 μL of phencyclidine-*d*₅ reference standard solution (100 μg/mL) into a 10-mL volumetric flask.
 - b. Dilute to volume with methanol and mix.
 - c. Store the working internal standard solution at 2 - 8 °C and protected from light.

XI. **CONTROL SAMPLES**

- A. Negative control urine – Horse urine sample demonstrated by analysis to contain no detectable nefopam or tramadol. Store at approximately - 20 °C.
- B. Positive control urine designated **PC** - Negative control urine supplemented with nefopam and tramadol, each at a concentration of 50 ng/mL. Pipette 5 μL of nefopam and tramadol working standard solutions (10 ng/μL) into a 16 x 100-mm tube. Add 1 mL of negative control horse urine. Cap and vortex-mix the contents of the tube. Prepare immediately before use.

XII. **SAMPLE REQUIREMENTS FOR ANALYSIS**

Prepare the following samples and standards for each analysis:

- A. Positive control designated **PC**; prepare the positive control at a concentration of 50 ng/mL from negative control horse urine and the nefopam and tramadol working standard solutions (10 ng/μL).
- B. Negative control sample designated **NC**; prepare negative control sample from negative control horse urine.

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- C. Test sample(s) designated **TS₁...TS_n**.
- D. Solvent blank designated **SB**.
- E. Standard mixture designated **S₁**.

XIII. CALIBRATOR AND SAMPLE PREPARATION

- A. Pipette 50 µL of phencyclidine-*d*₅ working internal standard solution (1.0 ng/µL) into each labeled 16 x 100-mm tube except those labeled **S₁** and **SB**. See Table 1.

NOTE: Prepare **S₁** and **SB** in steps XIV.N and XIV.D, respectively.

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

TUBE LABEL	Volume of Nefopam and Tramadol Working Standard Solutions (µL)	Volume of PCP- <i>d</i> ₅ Working Internal Standard Solution (µL)	Nefopam and Tramadol Injected into GC/MS (ng) (approximate)	PCP- <i>d</i> ₅ Injected into GC/MS (ng) (approximate)	Equivalent to Nefopam and Tramadol in the Urine (ng/mL)	Equivalent to PCP- <i>d</i> ₅ in the Urine (ng/mL)
PC	5.0	50	1.3	1.3	50	50
NC	0	50	0	1.3	0	50
TS₁...TS_n	0	50	unknown	1.3	unknown	50
SB	0	50	0	1.3	na	na
S₁	50	50	13	1.3	na	na

na = not applicable

- B. Add 2.0 mL of saturated sodium borate solution to each tube. Vortex-mix the contents of each tube for 3 - 5 seconds.
- C. Pipette 1.0 mL of negative control urine into the tube labeled **NC**.
- D. Pipette 1.0 mL of the positive control sample into the tube labeled **PC**.
- E. Pipette a 1.0-mL aliquot of the test sample(s) into the tube(s) labeled **TS₁...TS_n**.
- F. Vortex-mix the contents of each tube for 3 - 5 seconds.

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- G. Centrifuge the tubes at 1500 - 2000 rpm for approximately 5 minutes to remove sediment.

XIV. SOLID PHASE EXTRACTION OF NEFOPAM AND TRAMADOL

- A. Place a stopcock for each test sample and control sample 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 a 13 x 100-mm glass culture tube labeled **SB** in a collection rack position that will be used for a test sample. Collect 2 mL of elution solvent. Remove the tube and set aside until step XIV.O.
- 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 2 mL of methanol and 2 mL of water. Stop the flow as soon as each reagent reaches the top of the sorbent bed.
- F. Decant each prepared test sample and control sample into the corresponding column reservoirs and adjust the flows so that the solutions flow through the columns in not less than 1 minute.
- G. Rinse each column with 2 mL of water.
- H. Rinse each column with 2 mL of 0.1 *N* acetic acid.
- I. Dry the columns under full vacuum for approximately 4 minutes.
- J. Rinse each column with 2 mL of methanol.
- K. Dry the columns under full vacuum for approximately 2 minutes.
- L. Place labeled 13 x 100-mm glass culture tubes into position under the corresponding collection needles. Verify that the needles are positioned into the tubes.

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- M. Elute to collect with 1 mL of the elution solvent. Increase the vacuum briefly to draw a small volume of air through each column. Repeat with a second 1-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.
- N. Prepare the standard mixture, **S₁**, by adding 50 µL each of nefopam and tramadol working standard solutions (10 ng/µL) and 50 µL of phencyclidine-*d*₅ working internal standard solution (1.0 ng/µL) to a 13 x 100-mm glass culture tube labeled **S₁**.
- O. Pipette 50 µL of phencyclidine-*d*₅ internal standard solution (1.0 ng/µL) into the solution contained in tube **SB**.
- P. Evaporate the contents of each tube to dryness under nitrogen in an evaporator at 40 ± 5 °C.
- Q. Add 40 µL BSTFA to the contents of each tube. Sonicate for approximately 15 seconds and vortex-mix the contents for 5 – 10 seconds.
- R. Carefully transfer the entire contents of each tube to a low-volume insert in a labeled autosampler vial, using a new disposable pipette for each transfer.
- S. Cap and submit the vials for GC/MS analysis.

XV. **GAS CHROMATOGRAPHIC/MASS SPECTRAL IDENTIFICATION OF NEFOPAM
AND TRAMADOL**

- A. Gas Chromatograph and Mass Spectrometer Operating Parameters
 - 1. Instrumentation

Hewlett-Packard/Agilent model 5973 GC/MSD equipped with HP/Agilent MS Chemstation operating software (MS-Windows)
 - 2. GC column
 - a. Type: DB-5MS or DB-1MS (J&W Scientific, Agilent, or equivalent)
 - b. Length: 30 meters
 - c. Column i.d.: 0.25 mm

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- d. Film thickness: 0.25 μm
- 3. Carrier gas
 - a. Type: Helium (99.999%)
 - b. Flow rate: 1.0 mL/min
 - c. Column pressure: Set by electronic pressure control
- 4. Injection
 - a. Type: splitless
 - b. Injection volume: 1 μL
 - c. Purge on time: 0.4 minutes
- 5. Autosampler
 - a. Type: model 7683 (Agilent), or equivalent
 - b. Sample washes: 0
 - c. Sample pumps: 4
 - d. Viscosity delay: 0 seconds
 - e. Solvent washes: 6
- 6. Temperatures
 - a. Injector: 220 $^{\circ}\text{C}$
 - b. Oven temperature program: 120 $^{\circ}\text{C}$ (1.0 minute), 10 $^{\circ}\text{C}/\text{minute}$ to 280 $^{\circ}\text{C}$
 - c. Interface: 280 $^{\circ}\text{C}$
- 7. Ion source
 - a. Pressure: 5 - 8 x 10⁶ Torr
 - b. Temperature: 230 $^{\circ}\text{C}$
 - c. Ionization mode: electron-impact
- 8. Program: Selected Ion Monitoring
 - a. Name: NEFOSIM.M
 - b. Tune file: ATUNE.U
 - c. Start time: appropriate for the retention times of the compounds of interest

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- d. Dwell time: 20 msec
- e. EMV offset: appropriate for the assay, typically 0 V
- f. Ions: 166, 205, 246, and 248 amu for phencyclidine-*d*₅; 58, 165, 179, 195, 210, 225, 245, and 335 amu for nefopam and tramadol.

B. Procedure

Perform analyses in the order and with the method specified in Table 2:

Table 2. Order of analysis for identification of nefopam and tramadol in horse urine samples.

Run #	Vial	Method	Sample
1	1	NEFOSIM.M	PC
2	2	NEFOSIM.M	NC
3	3	NEFOSIM.M	TS ₁
⋮	⋮	⋮	⋮
n+2	n+2	NEFOSIM.M	TS _n
n+3	n+3	NEFOSIM.M	SB
n+4	n+4	NEFOSIM.M	S ₁

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 the analytes and internal standard, listed in Table 3, for each 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 test sample and the standard.
3. Calculate the relative ion area ratios for tramadol by dividing each

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qualifying ion area by the ion area of the most abundant ion as indicated in Table 3 for each test sample and the standard.

4. Calculate the relative ion area ratios for the internal standard by dividing the qualifying ion areas by the ion area of the more abundant ion as indicated in Table 3 for each test sample and the standard.

Table 3. Qualifying and quantifying ions for analysis of nefopam and tramadol in horse urine 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	58, <u>165</u> *, 179 , <u>225</u> *	179
Tramadol	58 , <u>245</u> , 335	58
Phencyclidine- <i>d</i> ₅	246, 205	205

* Either ion may be the least abundant ion.

5. Measure the signal-to-noise ratio for the least abundant qualifying ion for nefopam in the positive control sample and test sample(s). The least abundant qualifying ion is listed and underlined in Table 3.
6. Measure the signal-to-noise ratio for the least abundant qualifying ion for tramadol in the positive control sample and test sample(s). The least abundant qualifying ion is listed and underlined in Table 3.
7. Calculate the peak area ratio for nefopam by dividing the area of the nefopam quantifying ion (*i.e.*, A_{179}) at the retention time of nefopam by the area of the internal standard quantifying ion (*i.e.*, A_{205}) at the retention time of the internal standard for each test sample and the positive control sample.
8. Calculate the peak area ratio for tramadol by dividing the area of the tramadol quantifying ion (*i.e.*, A_{58}) at the retention time of tramadol by the area of the internal standard quantifying ion (*i.e.*, A_{205}) at the retention time of the internal standard for each test sample and the positive control sample.

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- D. Criteria for Identification of Nefopam and Tramadol in Urine Extracts
1. The retention times of the qualifying ions for nefopam and/or tramadol in each test sample must be within ± 0.05 minutes of the retention times of the same ions from the standard.
 2. The relative ion area ratios of the qualifying ions for nefopam and/or tramadol in each test sample must be within $\pm 20\%$ of the values of the same ions from the standard. The most abundant ion must be the ion indicated in Table 3.
 3. The signal-to-noise ratio of the least abundant qualifying ion for nefopam and/or tramadol in each test sample is greater than 3.
 4. The retention times of the qualifying ions for phencyclidine- d_5 in each test sample must be within ± 0.05 minutes of the retention times of the same ions from the standard.
 5. The relative ion area ratios of the qualifying ions for phencyclidine- d_5 from each test sample must be within $\pm 20\%$ of the values of the same ions from the standard. The more abundant ion must be the ion indicated in Table 3.
 6. 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 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

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to the trailing edge.

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 for the relevant analyte of the relevant test sample(s):

- A. The negative control sample or the solvent blank contains the analyte(s) as evidenced by the presence of the characteristic ions within the expected retention time window. In this instance, all test samples which would otherwise screen positive must be repeated.
- B. The positive control sample, **PC**, does not contain the analyte(s) according to the identification criteria specified for the test sample(s) as described in XV.D. In this instance, all test samples that would otherwise screen negative must be repeated.
- C. The internal standard ions are not detectable within the expected retention time window for the test sample(s).
- D. There is an interfering substance in the test sample(s). Refer to Section XVIII. **INTERFERING SUBSTANCES**.

XVII. CRITERIA FOR CONFIRMATORY ANALYSIS

Test samples which contain evidence of nefopam or tramadol according to the identification criteria described in XV.D should be submitted for confirmatory testing.

XVIII. INTERFERING SUBSTANCES

No known substances have been found to interfere with the determination of these analytes by this procedure. It is possible that a large amount of one or more substances in the sample could interfere with the extraction and chromatography of the analytes. This may be evident by a reduction in the response of the internal standard or by distorted peak shape for the analytes. Appropriate corrective actions would include modification of the extraction procedure to reduce the effect of interfering substances.

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XX. RESPONSIBLE PERSONNEL

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