

IDENTIFICATION AND DETERMINATION OF AMINOREX IN EXTRACTS OF HORSE URINE
BY LIQUID CHROMATOGRAPHY/MASS SPECTROMETRY

I. INTRODUCTION

Aminorex (Figure 1) is an illicit substance that was previously marketed in several European countries as Apiquel and Monocil® for use as an anorexic agent and appetite suppressant. However, this drug was withdrawn from the market after it was associated with several cases of pulmonary hypertension in human patients taking the drug. More recently, aminorex has been reported as a drug of abuse (Brewster *et al.*, 1991) that was sold as an alternative to methylaminorex (Figure 1).

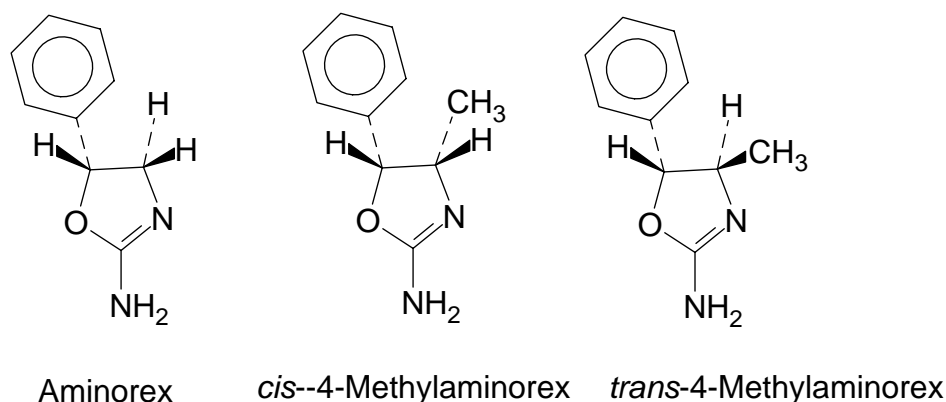


Figure 1. Chemical structures of aminorex, *cis*-4-methylaminorex, and *trans*-4-methylaminorex.

Aminorex is a central nervous system stimulant that produces effects similar to those of amphetamine in experimental animals and in humans. Aminorex is a controlled substance that has been assigned to Schedule I by the Drug Enforcement Administration because it has no medically accepted use and is subject to abuse. Furthermore, aminorex is classified by the Association of Racing Commissioners International, Inc. and the Ohio State Racing Commission as a class 1 substance. Therefore, the presence of aminorex in a test sample collected from a race horse is a violation of the rules of the Ohio State Racing Commission.

II. SCOPE OF METHOD

This standard operating procedure specifies procedures to be used to identify and determine aminorex from extracts of horse urine by liquid chromatography/mass spectrometry.

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III. REFERENCE PROCEDURES

- A. *OSU Analytical Toxicology Laboratory Standard Operating Procedures Manual Section 915 (“Agilent Model LC/MSD Trap Liquid Chromatograph/Mass Spectrometer Instrument Operating Procedure”)*
- B. *OSU Analytical Toxicology Laboratory Standard Operating Procedures Manual Section 713 (“Identification of Drugs and Drug Metabolites in Horse Urine by Gas Chromatography/Mass Spectrometry”)*
- C. *OSU Analytical Toxicology Laboratory Standard Operating Procedures Manual Section 714 (“Identification of Drugs and Drug Metabolites in Horse Urine by Liquid Chromatography/Mass Spectrometry”)*
- D. *OSU Analytical Toxicology Laboratory Standard Operating Procedures Manual Section 705 (“Identification of Sympathomimetic Amines in Horse Urine by Gas Chromatography/Mass Spectrometry”)*
- E. *OSU Analytical Toxicology Laboratory Standard Operating Procedures Manual Section 840 (“Identification of Aminorex in Extracts of Horse Urine by Gas Chromatography/Mass Spectrometry”)*
- F. *OSU Analytical Toxicology Laboratory Standard Operating Procedures Manual Section 841 (“Identification and Determination of Aminorex in Extracts of Horse Urine by Gas Chromatography/Mass Spectrometry”)*

IV. DEFINITIONS AND ACRONYMS

- A. ACS – American Chemical Society
- B. NCCLS – National Committee for Clinical Laboratory Standards
- C. ISTD – internal standard
- D. HPLC – high performance liquid chromatography or high performance liquid chromatograph
- E. LCMS – liquid chromatography/mass spectrometry or liquid chromatograph/mass spectrometer

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F. MSMS - a mass spectral technique in which an ion is formed, isolated, and then fragmented, resulting in a product ion mass spectrum

G. *m/z* – mass to charge ratio

V. PRINCIPLE OF METHOD

Aminorex and the internal standard (*cis*-4-methylaminorex) are extracted from horse urine under alkaline conditions into dichloromethane – isopropanol. The extracts are evaporated to dryness and the residues are dissolved in phosphate buffer prior to purification by solid phase extraction. The purified extracts are dissolved in aqueous formic acid and analyzed by liquid chromatography/mass spectrometry under positive ion electrospray chemical ionization MSMS conditions. The limit of detection of this method has not been rigorously determined but is less than 5 ng/mL of urine.

VI. 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 and reconstitution solutions (processed water from Barnstead Nanopure™ Infinity Deionization System, Barnstead International, Dubuque, Iowa 52001-1478, or cat. no. 365-4, Burdick & Jackson, Muskegon, MI 49442-6184, or equivalent)

B. Diluted ammonium hydroxide solution (1:1; v/v)

1. Reagents
 - a. Concentrated ammonium hydroxide, ACS reagent grade
 - b. Water
2. Procedure

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- a. **Caution: Prepare under a fume hood.**
 - b. Add one volume of concentrated ammonium hydroxide to an equal volume of water. Mix.
 3. Storage Requirements
 - a. Prepare the solution fresh daily.
 - b. Store at room temperature in a tightly closed glass container.
- C. 0.1 N Acetic acid reagent
 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.
- D. 0.1 M Potassium phosphate buffer (pH 9.0)
 1. Reagents
 - a. Potassium phosphate dibasic, ACS reagent grade
 - b. Water
 2. Procedure

Dissolve 17.4 g of potassium phosphate dibasic in sufficient water to produce 1000 mL of solution. Mix.

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3. Storage Requirements
 - a. Store at approximately 4 °C in a glass container.
 - b. Discard 1 year after preparation.

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

- F. Dichloromethane, ACS reagent grade or better (cat. no. 300-4, Burdick & Jackson, or equivalent)

- G. Isopropanol, ACS reagent grade or better (cat. no. 3032-08, Mallinckrodt Baker, Paris, KY 40361, or equivalent)

- H. Dichloromethane-isopropanol (3:1; v/v)
 1. Reagents
 - a. Dichloromethane
 - b. Isopropanol

 2. Procedure
 - a. **Caution: Prepare under a fume hood.**
 - b. Combine 3000 mL of dichloromethane and 1000 mL of isopropanol. Mix.

 3. Storage Requirements
 - a. Store at room temperature in a glass container.
 - b. Discard 1 year after preparation.

- I. Elution Solvent
 1. Reagents
 - a. Dichloromethane
 - b. Isopropanol
 - c. Concentrated ammonium hydroxide, ACS reagent grade

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2. Procedure
 - a. **Caution: Prepare under a fume hood.**
 - b. Add 2 mL of ammonium hydroxide to 20 mL of isopropanol. Mix.
 - c. Add 78 mL of dichloromethane while swirling. Mix.
3. Storage Requirements
 - a. Prepare the reagent fresh daily.
 - b. Store at room temperature in a tightly closed glass container.
- J. Formic acid, 88%, ACS reagent grade or better (cat. no. A118P-100, Fisher Scientific, Fair Lawn, NJ 07410, or equivalent)
- K. Acetonitrile, ACS reagent grade or better, suitable for HPLC applications (cat. no. 015-4, Burdick & Jackson, or equivalent)
- L. 0.1% Aqueous formic acid solution (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. Dilute to 1000 mL of solution and mix. Alternatively, add 4.0 mL of formic acid to 4L of water and 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 tightly closed glass container.
 - b. Discard 6 months after preparation.
- M. 0.1% Formic acid solution in acetonitrile (Mobile phase component "B")
 1. Reagents

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- 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. Dilute to 1000 mL of solution and mix. Alternatively, add 4.0 mL of formic acid to 4L of acetonitrile and mix.
 - c. Outgas by placing the container in an ultrasonic bath for approximately 2 minutes.
3. Storage Requirements
- a. Store at room temperature in a tightly closed glass container.
 - b. Discard 6 months after preparation.

VII. **SUPPLIES**

- A. 16 x 125-mm glass culture tubes with caps.
- B. 16 x 100-mm glass culture tubes.
- C. 13 x 100-mm glass, round-bottom or conical, culture tubes.
- D. Solid phase extraction columns, 3-mL, 30 mg, sorbent type DAU (cat. no. 532-DAU, Varian Consumable Products, Inc., Lake Forest, CA 92630)
- E. 2-mL glass autosampler vials (cat. no. C4011-2W, National Scientific, Duluth, GA 30097, or equivalent) and 11-mm aluminum seals with PTFE/silicone rubber septa (cat. no. C4011-4A, National Scientific, or equivalent). NOTE: Do not use PTFE/red rubber septa, e.g., Restek cat. no. 21175.
- F. Tissue paper wipers (e.g., Kimwipes®).
- G. Glass pasteur pipettes, disposable.
- H. Nitrogen gas.

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VIII. APPARATUS

A. Pipettes and tips.

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

1. 2.5-mL blunt-tip syringe and repetitive dispenser (gastight[®] syringe model no. 81416, dispenser model no. PB600-1, Hamilton Co., Reno, NV, 89502, or equivalent).
 2. Positive displacement pipette capable of measuring 1 - 10 μ L and tips (Gilson Microman Bio M10 and capillary/piston couplings CP10, Rainin Instrument Co., Inc., Woburn, MA 01888, or equivalent).
 3. Positive displacement pipette capable of measuring 10 - 100 μ L and tips (Gilson Microman Bio M100 and capillary/piston couplings CP100, Rainin Instrument Co., Inc., or equivalent).
 4. Positive displacement pipette capable of measuring 200 - 1000 μ L and tips (Gilson Microman Bio M1000 and capillary/piston couplings CP1000, Rainin Instrument Co., Inc., or equivalent).
 5. 10 - 100- μ L adjustable volume pipettor (Eppendorf 4810, Brinkmann Instruments Inc., Westbury, NY 11590, or equivalent).
 6. 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).
 7. 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).
- B. Vortex mixer (American Scientific Products, McGaw Park, IL 60085), Standard Multi-Tube Vortexer (cat. no. 58816-115, VWR International, Inc., West Chester, PA 19380), or equivalent.

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- C. pH meter (model Accumet[®] AR15 digital, Orion Research, Inc., Boston, MA, or equivalent).
- D. Rotorack (Glas-Col[®] Apparatus Co., Terre Haute, IN 47802, or equivalent).
- E. Centrifuge capable of centrifuging 16 x 125-mm culture tubes at 2000 - 3000 rpm (Damon/IEC division, model HN-S, Needham Heights, MA 02194, or equivalent).
- F. Evaporator capable of evaporating solvent to dryness from 16 x 100-mm and 13 x 100-mm culture 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 Consumable Products, or equivalent).
- H. Ultrasonic bath capable of sonicating mobile phase containers (Bransonic model 220, Branson Instruments Co., Shelton, CT, 06484, or equivalent).

IX. TEST SUBSTANCE

The test substance specified in this procedure is horse urine.

X. VOLUME REQUIRED

Analyze duplicate 1-mL aliquots of undiluted test sample (2 mL total) if the estimated concentration of the analyte in the test sample is between 5 and 300 ng/mL. If the estimated concentration of an analyte is greater than 300 ng/mL, prepare a dilution of an aliquot of the test sample with negative control horse urine so that the approximate concentration of that analyte is between 5 and 300 ng/mL.

XI. REFERENCE STANDARD SOLUTIONS

- A. Aminorex reference standard solution “**A**” – nominally 1.0 mg/mL in acetonitrile (cat. no. A-040, Cerilliant Corp., Austin, TX 78720-1088, or equivalent.). Store the standard solution at approximately -15 °C and protected from light.
- B. Aminorex reference standard solution “**B**” – nominally 1.0 mg/mL in acetonitrile (cat. no. FSU-099-1000, Cambridge Isotope Laboratories, Inc., Andover, MA 01810-5413, or equivalent). Store the standard solution at approximately -15 °C and protected from light.

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- C. *cis*-4-Methylaminorex reference standard solution – nominally 1.0 mg/mL in acetonitrile (cat. no. M-045, Cerilliant Corp., or equivalent.). Store the standard solution at approximately -15 °C and protected from light.

XII. INTERMEDIATE AND WORKING STANDARD SOLUTIONS

- A. Aminorex Intermediate Standard Solution “**A**” – 100 ng/μL
1. Reagents
 - a. Aminorex reference standard solution “**A**” (1.0 mg/mL)
 - b. Acetonitrile
 2. Procedure
 - a. Pipette 1.0 mL of aminorex reference standard solution “**A**” (1.0 mg/mL) into a 10-mL volumetric flask.
 - b. Dilute to volume with acetonitrile and mix by inversion.
 3. Storage Requirements
 - a. Store the intermediate standard solution at approximately -15 °C and protected from light.
 - b. Discard according to the expiration date for the reference standard solution or 1 year after preparation, whichever is sooner.
 4. Use aminorex intermediate standard solution “**A**” (100 ng/μL) to prepare aminorex working standard solution “**A**” (10 ng/μL).
- B. Aminorex Working Standard Solution “**A**” – 10 ng/μL
1. Reagents
 - a. Aminorex intermediate standard solution “**A**” (100 ng/μL)
 - b. Acetonitrile
 2. Procedure
 - a. Pipette 1.0 mL of aminorex intermediate standard solution “**A**”

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- (100 ng/ μ L) into a 10-mL volumetric flask.
 - b. Dilute to volume with acetonitrile and mix by inversion.
 3. Storage Requirements
 - a. Store the working standard solution at approximately -15 °C and protected from light.
 - b. Discard according to the expiration date for aminorex intermediate standard solution "A" (100 ng/ μ L).
 4. Use aminorex working standard solution "A" (10 ng/ μ L) to prepare the aminorex working standard solution "A" (1.0 ng/ μ L) and calibrators **C₅**, **C₆**, and **C₇**.
- C. Aminorex Working Standard Solution "A" – 1.0 ng/ μ L
1. Reagents
 - a. Aminorex working standard solution "A" (10 ng/ μ L)
 - b. Acetonitrile
 2. Procedure
 - a. Pipette 1.0 mL of aminorex working standard solution "A" (10 ng/ μ L) into a 10-mL volumetric flask.
 - b. Dilute to volume with acetonitrile and mix by inversion.
 3. Storage Requirements
 - a. Store the working standard solution at approximately -15 °C and protected from light.
 - b. Discard according to the expiration date for aminorex working standard solution "A" (10 ng/ μ L).
 4. Use aminorex working standard solution "A" (1.0 ng/ μ L) to prepare the standard, **S₁** and calibrators **C₁**, **C₂**, **C₃**, and **C₄**.
- D. Aminorex Working Standard Solution "B" – 100 ng/ μ L
1. Reagents

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- a. Aminorex reference standard solution "**B**" (1.0 mg/mL)
 - b. Acetonitrile
 2. Procedure
 - a. Pipette 1.0 mL of aminorex reference standard solution "**B**" (1.0 mg/mL) into a 10-mL volumetric flask.
 - b. Dilute to volume with acetonitrile and mix by inversion.
 3. Storage Requirements
 - a. Store the working standard solution at approximately -15 °C and protected from light.
 - b. Discard according to the expiration date for the reference standard solution or 1 year after preparation, whichever is sooner.
 4. Use aminorex working standard solution "**B**" (100 ng/μL) to prepare aminorex working standard solution "**B**" (10 ng/μL) and positive control sample **PC**₂.
- E. Aminorex Working Standard Solution "**B**" – 10 ng/μL
1. Reagents
 - a. Aminorex working standard solution "**B**" (100 ng/μL)
 - b. Acetonitrile
 2. Procedure
 - a. Pipette 1.0 mL of aminorex working standard solution "**B**" (100 ng/μL) into a 10-mL volumetric flask.
 - b. Dilute to volume with acetonitrile and mix by inversion.
 3. Storage Requirements
 - a. Store the working standard solution at approximately -15 °C and protected from light.
 - b. Discard according to the expiration date for aminorex working standard solution "**B**" (100 ng/μL).

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4. Use aminorex working standard solution “**B**” (10 ng/μL) to prepare the positive control sample **PC₁**.
- F. *cis*-4-Methylaminorex Intermediate Standard Solution – 100 ng/μL
1. Reagents
 - a. *cis*-4-Methylaminorex reference standard solution (1.0 mg/mL)
 - b. Acetonitrile
 2. Procedure
 - a. Pipette 1.0 mL of *cis*-4-methylaminorex reference standard solution (1.0 mg/mL) into a 10-mL volumetric flask.
 - b. Dilute to volume with acetonitrile and mix by inversion.
 3. Storage Requirements
 - a. Store the intermediate standard solution at approximately -15 °C and protected from light.
 - b. Discard according to the expiration date for the reference standard solution or 1 year after preparation, whichever is sooner.
- G. *cis*-4-Methylaminorex Working Internal Standard Solution – 10 ng/μL
1. Reagents
 - a. *cis*-4-Methylaminorex intermediate standard solution (100 ng/μL)
 - b. Acetonitrile
 2. Procedure
 - a. Pipette 1.0 mL of *cis*-4-methylaminorex intermediate standard solution (100 ng/μL) into a 10-mL volumetric flask.
 - b. Dilute to volume with acetonitrile and mix by inversion.
 3. Storage Requirements
 - a. Store the working standard solution at approximately -15 °C and

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- protected from light.
- b. Discard according to the expiration date for the intermediate standard solution.

XIII. CONTROL SAMPLES

- A. Negative control urine - Horse urine demonstrated by analysis to contain no detectable aminorex and *cis*-4-methylaminorex. Store the negative control urine at approximately -15 °C.
- B. Positive control urine designated **PC₁** – Negative control urine supplemented with aminorex at a concentration of 40 ng/mL. Pipette 100 µL of aminorex working standard solution “**B**” (10 ng/µL) into a 25-mL volumetric flask. Dilute to volume with negative control urine. Cap and mix by rotation. Store the positive control urine at 2 - 8 °C. Discard 5 days after preparation.
- C. Positive control urine designated **PC₂** – Negative control urine supplemented with aminorex at a concentration of 240 ng/mL. Pipette 60 µL of aminorex working standard solution “**B**” (100 ng/µL) into a 25-mL volumetric flask. Dilute to volume with negative control urine. Cap and mix by rotation. Store the positive control urine at 2 - 8 °C. Discard 5 days after preparation.

XIV. SAMPLE REQUIREMENTS FOR ANALYSIS

Prepare the following samples and standards for each analysis:

- A. Calibrators designated **C₁**, **C₂**, **C₃**, **C₄**, **C₅**, **C₆**, and **C₇**; prepare calibrators at concentrations of 5.0, 10, 20, 50, 100, 200, and 300 ng/mL from negative control urine and aminorex working standard solutions “**A**” (1.0 and 10 ng/µL).
- B. System washes designated **SYS₁** and **SYS₂**; prepare the system washes from 0.1% aqueous formic acid and acetonitrile.
- C. Negative control sample designated **NC**; prepare negative control sample from negative control urine.
- D. Test sample designated **TS_{1a}** and **TS_{1b}**; **a** and **b** are designations for test sample replicates.
- E. Solvent blanks designated **SB_{1a}** and **SB_{1b}**; **a** and **b** are designations for solvent

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blank replicates.

- F. Positive control samples designated **PC_{1a}**, **PC_{1b}**, **PC_{2a}**, and **PC_{2b}**; **a** and **b** are designations for control sample replicates; prepare positive control samples from negative control horse urine and working standard solutions “**B**” (10 and 100 ng/μL).
- G. Standard mixture designated **S₁**.

XV. CALIBRATOR AND SAMPLE PREPARATION

- A. Pipette 10 μL of *cis*-4-methylaminorex working standard solution (10 ng/μL) into each labeled 16 x 125-mm test tube except those labeled **SYS₁**, **SYS₂**, **SB_{1a}**, **SB_{1b}**, and **S₁**.

NOTE: Prepare **SYS₁** and **SYS₂**, in step XVIII.Q, **SB_{1a}** and **SB_{1b}** in step XVIII.D, and **S₁** in step XVIII.N, respectively.

- B. Pipette 5.0, 10, 20, and 50 μL of aminorex working standard solution “**A**” (1.0 ng/μL) into the tubes labeled **C₁**, **C₂**, **C₃**, and **C₄**, respectively. See Table 1.
- C. Pipette 10, 20, and 30 μL of aminorex working standard solution “**A**” (10 ng/μL) into the tubes labeled **C₅**, **C₆**, and **C₇**, respectively. See Table 1.

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

TUBE LABEL	Volume of Aminorex Working Standard Solution (μ L)	Volume of <i>cis</i> -4-Methylaminorex Working Internal Standard Solution (μ L)	Aminorex Injected into LCMS, (ng)	<i>Cis</i> -4-Methylaminorex Injected into LCMS, (ng)	Equivalent to Aminorex in the Urine, ng/mL	Equivalent to <i>cis</i> -4-Methylaminorex in the Urine, ng/mL
C₁	5.0 ¹	10	0.050	1.0	5.0	100
C₂	10 ¹	10	0.10	1.0	10	100
C₃	20 ¹	10	0.20	1.0	20	100
C₄	50 ¹	10	0.50	1.0	50	100
C₅	10 ²	10	1.0	1.0	100	100
C₆	20 ²	10	2.0	1.0	200	100
C₇	30 ²	10	3.0	1.0	300	100
SYS_{1..2}	0	10	0	0	na	na
NC	0	10	0	1.0	0	100
TS_{1a-1b}	0	10	unknown	1.0	unknown	100
SB_{1a-1b}	0	10	0	0	na	na
PC_{1a-1b}	100 ³	10	0.40	1.0	40	100
PC_{2a-2b}	60 ⁴	10	2.4	1.0	240	100
S₁	100 ¹	10	1.0	1.0	na	na

na = not applicable

¹ – volume of aminorex working standard solution “A” (1.0 ng/ μ L)

² – volume of aminorex working standard solution “A” (10 ng/ μ L)

³ – volume of aminorex working standard solution “B” (10 ng/ μ L) used to prepare 25 mL of **PC₁**.

⁴ – volume of aminorex working standard solution “B” (100 ng/ μ L) used to prepare 25 mL of **PC₂**.

- D. Pipette 1.0 mL of negative control urine into the tubes labeled **NC**, **C₁**, **C₂**, **C₃**, **C₄**, **C₅**, **C₆**, and **C₇**.
- E. Pipette 1.0-mL aliquots of the test sample into the tubes labeled **TS_{1a}** and **TS_{1b}** if the estimated concentration of the analyte in the test sample is between 5 and 300 ng/mL. If the estimated concentration of the analyte is greater than 300 ng/mL, prepare an appropriate dilution of an aliquot of the test sample with negative control horse urine and pipette 1.0-mL aliquots of the diluted sample into the tubes labeled **TS_{1a}** and **TS_{1b}**.

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- F. Pipette 1.0 mL of positive control urine, **PC₁**, into each of the tubes labeled **PC_{1a}** and **PC_{1b}**.
- G. Pipette 1.0 mL of positive control urine, **PC₂**, into each of the tubes labeled **PC_{2a}** and **PC_{2b}**.

XVI. LIQUID - LIQUID EXTRACTION

- A. Pipette 100 μ L of diluted ammonium hydroxide solution into each tube.
- B. Pipette 5 mL of dichloromethane-isopropanol (3:1; v/v) reagent into each tube.
- C. Cap the tubes; mix by end-over-end rotation at 5 - 20 rpm for 5 - 10 minutes.
- D. Centrifuge the tubes at 2000 - 3000 rpm for 5 minutes or until the phases have separated.
- E. Remove and discard the top layers by aspiration.
- F. Decant the bottom layers into new labeled 16 x 100-mm tubes.
- G. Evaporate the contents of each tube to dryness under nitrogen in an evaporator at $40 \pm 5^\circ\text{C}$.

XVII. SAMPLE PREPARATION FOR SOLID PHASE EXTRACTION

- A. Dissolve the residue from each extract in 100 μ L of methanol. Vortex-mix the contents of each tube for 3 - 5 seconds.
- B. Add 3 mL of 0.1 M potassium phosphate buffer (pH 9.0) to each tube.
- C. Vortex-mix the contents of each tube for 5 - 20 seconds.

XVIII. SOLID PHASE EXTRACTION

- 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.

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- 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 culture tubes labeled **SB_{1a}** and **SB_{1b}** in the collection rack positions that will be used for the corresponding test sample replicates. Collect 2 mL of the elution solvent. Remove the tubes and save until step XVIII.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 1 mL of methanol, 1 mL of water, and 1 mL of 0.1 M potassium phosphate dibasic buffer (pH 9.0). Stop the flow as soon as each reagent reaches the top of the sorbent bed.
- F. Decant each solution into the corresponding column reservoir and adjust the flows so that the solutions flow through the columns in not less than 2 minutes.
- G. Rinse each column with 1 mL of water.
- H. Rinse each column with 1 mL of 0.1 N acetic acid solution.
- I. Dry the columns under full vacuum for 1 minute.
- J. Rinse each column with 1 mL of methanol.
- K. Dry the columns under full vacuum for 1 minute.
- L. Place labeled 13 x 100-mm culture tubes into position under the corresponding collection needles. Verify that the needles are positioned into the tubes.
- M. Elute to collect with 1 mL of the elution solvent. Briefly increase the vacuum to remove all elution solvent from the sorbent bed and then repeat with a second 1-mL aliquot of elution solvent. Verify that all the elution solvent has been drawn through the columns and then briefly increase the vacuum to remove all elution solvent from the sorbent bed.

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- N. Prepare the standard, **S**₁, by pipetting 100 μ L of aminorex working standard solution "**A**" (1.0 ng/ μ L) and 10 μ L of *cis*-4-methylaminorex working internal standard solution (10 ng/ μ L) into a 13 x 100-mm culture tube labeled **S**₁.
- O. Pipette 10 μ L of *cis*-4-methylaminorex working internal standard solution (10 ng/ μ L) into the solution contained in tubes **SB**_{1a} and **SB**_{1b}.
- P. Evaporate the contents of each tube to dryness under nitrogen in a water bath at 40 \pm 5 $^{\circ}$ C.
- Q. Prepare the system wash tubes by labeling two 13 x 100-mm culture tubes **SYS**₁ and **SYS**₂.
- R. Add 60 μ L of acetonitrile to each of the tubes. Vortex-mix the contents of each tube for 10 - 20 seconds.
- S. Add 440 μ L of 0.1% aqueous formic acid to each of the tubes. Vortex-mix the contents of each tube for 10 - 20 seconds.
- T. Carefully transfer the entire contents of each tube to an autosampler vial, using a new disposable pipette for each transfer.
- U. Cap and submit the vials for LCMS analysis.

XIX. LIQUID CHROMATOGRAPHIC/MASS SPECTRAL IDENTIFICATION OF AMINOREX

- A. Liquid Chromatograph and Mass Spectrometer Operating Parameters
 - 1. Instrumentation:
 - a. Agilent LC/MSD-Trap equipped with Agilent MS Chemstation operating software
 - 2. LC column:
 - a. Guard column: Metaguard™ Polaris™ C18-A (cat. no. 2000-MG2 Varian Consumable Products)
 - b. Column type: Metasil™ Polaris C18-A, (cat. no. 2000-100X030, Varian)

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- c. Column length: 100 mm
d. Column i.d.: 3.0 mm
e. Particle size: 5 µm
3. Chromatography:
- a. Program name: AMINOREX.M
b. Mobile phase:
i. Component "A" 0.1% aqueous formic acid
ii. Component "B" 0.1% formic acid in acetonitrile
c. Gradient and flow rate:
- | Time (min) | % B | Flow |
|------------|-----|------|
| 0.00 | 15% | 0.30 |
| 0.01 | 20% | 0.30 |
| 2.00 | 20% | 0.30 |
| 5.00 | 60% | 0.30 |
| 6.00 | 90% | 0.30 |
| 8.00 | 90% | 0.30 |
| 8.01 | 15% | 0.60 |
| 12.00 | 15% | 0.60 |
- d. Column temperature: 35 °C
e. Stop time: 12 minutes
f. Injection volume: 5 µL
g. Overlapped injection: disabled
h. Solvent divert time: 2.5 minutes
4. Ionization:
- a. Type: electrospray, positive ion
b. Resolution: normal
c. Nebulizer pressure: 40 psi
d. Dry gas temperature: 365 °C
e. Dry gas flow rate: 9 L/min
5. Tune parameters:
- a. Capillary voltage: -3500V
b. Skimmer voltage: 30V
c. Capillary exit: 90V
d. Octapole 1 voltage: 11V
e. Octapole 2 voltage: 2.0V

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- f. Octapole RF voltage: 100Vpp
- g. Trap drive: 28 (arbitrary units)
- h. Lens 1 voltage: -5.0V
- i. Lens 2 voltage: -60.0V
- j. Maximum accumulation: 50 msec
- k. ICC target: 30000
- l. Scan range: 50 – 250 amu
- m. Scan averages: 5

6. MSMS program – aminorex

- a. Transition: *m/z* 163.0 → scan
- b. Isolation width: *m/z* 1.3
- c. Fragmentation cutoff: *m/z* 85
- d. Fragmentation amplitude: 0.80 V (SmartFrag off)
- e. Fragmentation width: 4 amu
- f. Product ions: *m/z* 120, 163

7. MSMS program – 4-*cis*-methylaminorex

- a. Transition: *m/z* 177.0 → scan
- b. Isolation width: *m/z* 2.0
- c. Fragmentation cutoff: *m/z* 95
- d. Fragmentation amplitude: 0.80 V (SmartFrag off)
- e. Fragmentation width: 4 amu
- f. Product ions: *m/z* 134, 177

B. Procedure

Perform analyses in the order and with the acquisition method specified in Table 2.

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Table 2. Run number, vial number, acquisition method, and sample designation for LCMS analysis for identification and determination of aminorex from horse urine.

Run #	Vial	Method	Sample
1-7	1-7	AMINOREX.M	C ₁ , C ₂ , C ₃ , C ₄ , C ₅ , C ₆ , C ₇
8-9	8-9	AMINOREX.M	SYS ₁ , SYS ₂
10-11	10-11	AMINOREX.M	PC _{1a} , PC _{1b}
12-13	12-13	AMINOREX.M	PC _{2a} , PC _{2b}
14	14	AMINOREX.M	NC
15	15	AMINOREX.M	TS _{1a}
16	16	AMINOREX.M	SB _{1a}
17	17	AMINOREX.M	TS _{1b}
18	18	AMINOREX.M	SB _{1b}
19-20	10-11	AMINOREX.M	PC _{1a} , PC _{1b}
21-22	12-13	AMINOREX.M	PC _{2a} , PC _{2b}
23	19	AMINOREX.M	S ₁

Analyze additional test sample extracts and solvent blanks by duplicating the specified sequence indicated, inserting the additional sample extracts in runs 15-18.

C. Evaluation of Mass Spectral Data for Aminorex

1. Obtain the summed ion chromatograms, the extracted ion chromatograms and retention times for the qualifying ions for the analyte and internal standard listed in Table 3 for each test sample replicate extract, calibrator extract, control sample replicate extract, and the standard.
2. Calculate the relative ion area ratios for aminorex by dividing each qualifying ion area by the ion area of the more abundant qualifying ion as indicated in Table 3 for each replicate of the test sample and the standard.
3. Calculate the relative ion area ratios for *cis*-4-methylaminorex by dividing each qualifying ion area by the ion area of the more abundant qualifying ion as indicated in Table 3 for each replicate of the test sample and the standard.

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standard.

Table 3. Qualifying and quantifying ions for LCMS analysis for aminorex in horse urine; the more abundant qualifying ions are indicated in **bold** type and the less abundant qualifying ions are underlined.

Analyte (in order of elution)	Qualifying Ions (m/z)	Quantifying Ions, (m/z)
Aminorex	163→ 120 , <u>163</u>	120+163 (summed)
<i>cis</i> -4-Methylaminorex (ISTD)	177→ 134 , <u>177</u>	134+177 (summed)

4. Calculate the peak area ratio for aminorex by dividing the summed area of the quantifying ions at the retention time of aminorex by the summed area of the quantifying ions at the retention time of *cis*-4-methylaminorex for each calibrator, test sample replicate, and control sample replicate.
5. Measure the signal-to-noise ratio of the less abundant qualifying ion as indicated in Table 3 at the retention time of aminorex for each replicate of the test sample.
6. Obtain and print the MSMS spectrum of aminorex from the standard data file. At the retention time of aminorex, select and print the MSMS spectrum from the negative control sample extract, test sample replicate extracts, and solvent blanks from the corresponding data files.

D. Criteria for Identification of Aminorex from Urine Extracts

1. The retention times of the qualifying ions for aminorex in both test sample replicates must be within $\pm 2\%$ of the retention times of the same ions from the standard.
2. The retention times of the qualifying ions for the internal standard in both test sample replicates must be within $\pm 2\%$ of the retention time of the same ions from the standard.
3. The relative ion area ratios of the qualifying ions for aminorex in both test sample replicates must be within $\pm 30\%$ of the values of the same ions from the standard. The more abundant ion must be the qualifying ion indicated in Table 3.

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4. The relative ion area ratios of the qualifying ions for the internal standard in both test sample replicates must be within $\pm 30\%$ of the values of the same ions from the standard. The more abundant ion must be the qualifying 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 width of the peak at its base should be less than 0.50 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 to the trailing edge.
 6. The MSMS spectra of aminorex from both test sample replicates and from the standard must have essentially the same fragmentation patterns.
- E. Determination of the Concentration of Aminorex in Urine
1. Plot the peak area ratios of the summed quantifying ions for each calibrator versus the concentration of aminorex in the calibrator. Perform linear regression analysis on these data to obtain the slope, intercept, and correlation coefficient of the standard curve for aminorex.
 2. Calculate the concentration of aminorex in each test sample and positive control sample 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.

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Concentration = Calculated concentration / dilution factor

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

Average concentration = $\frac{1}{2}$ (concentration **TS_{1a}** + concentration **TS_{1b}**)

XX. 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 for the relevant analyte:

- A. The negative control sample or the solvent blanks contain aminorex as evidenced by the presence of the characteristic ions within the expected retention time window.
- B. The standard curve for aminorex has a correlation coefficient less than 0.98.
- C. The internal standard ions are not detectable within the expected retention time window for either test sample replicate.
- D. The measured concentration of aminorex in a positive control sample replicate differs from the nominal concentration by more than 30% for either **PC₁** or **PC₂**.
- E. The measured concentration of aminorex in a positive control sample replicate differs from the corresponding average concentration by more than 15% for either **PC₁** or **PC₂**.
- F. The peak area ratio of aminorex in any test sample replicate is greater than the peak area ratio for aminorex in the highest calibrator; dilute an aliquot of the test sample as described in section X of this procedure and repeat the analysis.
- 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(s). Refer to Section XXII. **INTERFERING SUBSTANCES.**

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XXI. CRITERIA FOR REPORTING A SAMPLE POSITIVE FOR AMINOREX

Report a sample positive for aminorex when all of the following criteria have been met:

- A. The test sample contains aminorex according to the criteria described in XIX.D.
- B. The peak area ratio of aminorex in each replicate of the test sample is greater than the peak area ratio of aminorex in the lowest calibrator.
- C. The signal-to-noise ratio of the less abundant qualifying ion for aminorex in each replicate of the test sample is greater than 10.
- D. The presence of aminorex in the test sample has been demonstrated by a second analytical methodology, e.g., gas chromatography/mass spectrometry.

XXII. INTERFERING SUBSTANCES

No known substances have been found to interfere with the determination of the analyte by this procedure. It is possible that a large amount of one or more substances in the extract could cause increased background signal levels or suppress the ionization of the analyte or the internal standard. This would be evident by a decrease in the signal-to-noise ratio for a given ion channel or 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 the use of a different chromatographic system to change or improve the separation.

XXIII. REFERENCES

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XXIV. RESPONSIBLE PERSONS

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

**STANDARD OPERATING PROCEDURES
OSU ANALYTICAL TOXICOLOGY LABORATORY**

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