

IDENTIFICATION OF AMINOEX IN EXTRACTS OF HORSE PLASMA 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 However, this drug was withdrawn from the market after it was associated with several cases of pulmonary hypertension in human patients taking the drug.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).

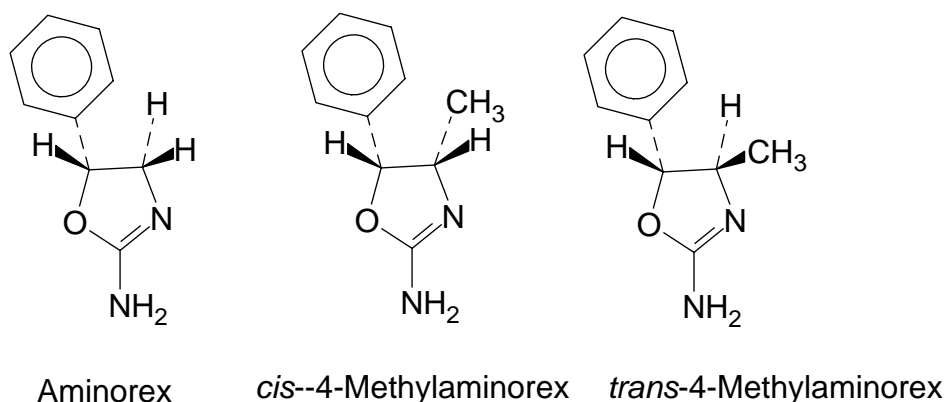


Figure 1. Chemical structures of aminorex, *cis*-4-methylaminorex, and *trans*-4-methylaminorex.

Aminorex is a central nervous system stimulant that produces effects similar to those of amphetamine in experimental animals and in humans. Aminorex has been assigned to Schedule I by the Drug Enforcement Administration because it has no medically accepted use and is subject to abuse by humans. 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 aminorex from extracts of horse plasma 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 841 (“Identification and Determination of Aminorex in Extracts of Horse Urine by Gas Chromatography/Mass Spectrometry”)*
- F. *OSU Analytical Toxicology Laboratory Standard Operating Procedures Manual Section 840 (“Identification of Aminorex in Extracts of Horse Urine by Gas Chromatography/Mass Spectrometry”)*
- G. *OSU Analytical Toxicology Laboratory Standard Operating Procedures Manual Section 641 (“Identification and Determination of Aminorex in Extracts of Horse Urine by Liquid 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

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chromatograph

- E. LCMS – liquid chromatography/mass spectrometry or liquid chromatograph/mass spectrometer
- 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 plasma by solid phase extraction. The extracts are dissolved in acetonitrile and 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 approximately 1 ng/mL of plasma.

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. 0.1 N Acetic acid reagent
 - 1. Reagents
 - a. Glacial acetic acid, ACS reagent grade
 - b. Water

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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. 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.
 3. Storage Requirements
 - a. Store at approximately 4 °C in a glass container.
 - b. Discard 1 year after preparation.
- D. Methanol, ACS reagent grade or better (cat. no. 230-4, Burdick & Jackson, or equivalent)
- E. Dichloromethane, ACS reagent grade or better (cat. no. 300-4, Burdick & Jackson, or equivalent)
- F. Isopropanol, ACS reagent grade or better (cat. no. 3032-08, Mallinckrodt Baker, Paris, KY 40361, or equivalent)
- G. Elution Solvent

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1. Reagents
 - a. Dichloromethane
 - b. Isopropanol
 - c. Concentrated ammonium hydroxide, ACS reagent grade
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.
- H. Formic acid, 88%, ACS reagent grade or better (cat. no. A118P-100, Fisher Scientific, Fair Lawn, NJ 07410, or equivalent)
- I. Acetonitrile, ACS reagent grade or better, suitable for HPLC applications (cat. no. 015-4, Burdick & Jackson, or equivalent)
- J. 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

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- a. Store at approximately 4 °C in a tightly closed glass container.
 - b. Discard 6 months after preparation.
- K. 0.1% Formic acid solution in acetonitrile (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. 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 100-mm glass culture tubes.
- B. 13 x 100-mm glass conical tubes.
- C. Solid phase extraction columns, 3-mL, 30 mg, sorbent type DAU (cat. no. 532-DAU, Varian Consumable Products, Inc., Lake Forest, CA 92630).
- D. 2-mL glass autosampler vials (cat. no. C4011-2W, National Scientific, Duluth, GA 30097, or equivalent) with 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.

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- E. Tissue paper wipers (e.g., Kimwipes®).
- F. Glass pasteur pipettes, disposable.
- G. Nitrogen gas.

VIII. **APPARATUS**

- A. Pipettes and tips.

Note: Use positive displacement pipettes to pipette all standard solutions. Plasma 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).

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- 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.
- C. pH meter (model Accumet[®] AR15 digital, Orion Research, Inc., Boston, MA, or equivalent).
- D. Solid phase extraction manifold (Varian Vac Elut SPS 24[™] cat. no. 1223-4022, Varian Consumable Products, or equivalent).
- E. Evaporator capable of evaporating solvent to dryness from 13 x 100-mm conical tubes at 40 ± 5°C under nitrogen (The Meyer N-Evap, Organomation Assoc. Inc., South Berlin, MA 01549, or equivalent).
- F. 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 plasma.

X. VOLUME REQUIRED

Analyze a 1.0-mL aliquot of undiluted test sample.

XI. REFERENCE STANDARD SOLUTIONS

- A. Aminorex reference standard solution – 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. *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 – 100 ng/μL

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1. Reagents
 - a. Aminorex reference standard solution (1.0 mg/mL)
 - b. Acetonitrile
 2. Procedure
 - a. Pipette 1.0 mL of aminorex 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.
 4. Use aminorex intermediate standard solution (100 ng/ μ L) to prepare aminorex intermediate standard solution (10 ng/ μ L).
- B. Aminorex Intermediate Standard Solution – 10 ng/ μ L
1. Reagents
 - a. Aminorex intermediate standard solution (100 ng/ μ L)
 - b. Acetonitrile
 2. Procedure
 - a. Pipette 1.0 mL of aminorex intermediate standard solution (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 intermediate standard solution at approximately -15 °C and protected from light.
 - b. Discard according to the expiration date for aminorex intermediate standard solution (100 ng/ μ L).

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4. Use aminorex intermediate standard solution (10 ng/ μ L) to prepare the aminorex intermediate standard solution (1.0 ng/ μ L).
- C. Aminorex Intermediate Standard Solution – 1.0 ng/ μ L
1. Reagents
 - a. Aminorex intermediate standard solution (10 ng/ μ L)
 - b. Acetonitrile
 2. Procedure
 - a. Pipette 1.0 mL of aminorex intermediate standard solution (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 intermediate standard solution at approximately -15 °C and protected from light.
 - b. Discard according to the expiration date for aminorex intermediate standard solution (10 ng/ μ L).
 4. Use aminorex intermediate standard solution (1.0 ng/ μ L) to prepare aminorex working standard solution (0.10 ng/ μ L).
- D. Aminorex Working Standard Solution – 0.10 ng/ μ L
1. Reagents
 - a. Aminorex intermediate standard solution (1.0 ng/ μ L)
 - b. Acetonitrile
 2. Procedure
 - a. Pipette 1.0 mL of aminorex intermediate standard solution (1.0 ng/ μ L) into a 10-mL volumetric flask.
 - b. Dilute to volume with acetonitrile and mix by inversion.

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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 (1.0 ng/μL).
 4. Use aminorex working standard solution (0.10 ng/μL) to prepare the standard, **S₁**, and positive control, **PC**.
- E. *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.
- F. *cis*-4-Methylaminorex Intermediate 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

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- b. solution (1.0 ng/ μ L) into a 10-mL volumetric flask.
Dilute to volume with acetonitrile and mix by inversion.

3. Storage Requirements

- a. Store the internal standard solution at approximately -15 °C and protected from light.
- b. Discard according to the expiration date for the intermediate standard solution.

XIII. CONTROL SAMPLES

- A. Negative control plasma - Horse plasma demonstrated by analysis to contain no detectable aminorex and *cis*-4-methylaminorex. Store the negative control plasma at approximately -15 °C.
- B. Positive control plasma designated **PC** – Negative control plasma supplemented with aminorex at a concentration of 2 ng/mL. Preparation of the positive control sample is described in Section XV.

XIV. SAMPLE REQUIREMENTS FOR ANALYSIS

Prepare the following samples and standards for each analysis:

- A. Positive control sample designated **PC**; prepare the positive control sample from negative control plasma and the aminorex working standard solution (0.10 ng/ μ L).
- 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 plasma.
- D. Test sample designated **TS₁**.
- E. Solvent blank designated **SB₁**.
- F. Standard mixture designated **S₁**.

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XV. CALIBRATOR AND SAMPLE PREPARATION

- A. Pipette 50 μ L of *cis*-4-methylaminorex internal standard solution (0.1 ng/ μ L) into each labeled 16 x 100-mm test tube except those labeled **SYS₁**, **SYS₂**, **SB₁**, and **S₁**.

NOTE: Prepare **SYS₁** and **SYS₂**, in step XVI.Q, **SB₁** in step XVI.D, and **S₁** in step XVI.N, respectively.

- B. Pipette 20 μ L of aminorex working standard solution (0.10 ng/ μ L) into the tube labeled **PC**. See Table 1.

Table 1. Volumes of working standard solutions required to prepare 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 Plasma, ng/mL	Equivalent to <i>cis</i> -4-Methylaminorex in the Plasma, ng/mL
PC	20	50	1.5	3.8	2.0	5.0
SYS₁₋₂	0	0	0	0	na	na
NC	0	50	0	3.8	0	5.0
TS₁	0	50	unknown	3.8	unknown	5.0
SB₁	0	50	0	3.8	na	na
S₁	150	150	3.8	3.8	na	na

na = not applicable

- C. Add 2 mL of 0.1 M potassium phosphate buffer (pH 9.0) to each tube. Vortex-mix the contents of each tube for 5 - 10 seconds.
- D. Pipette 1.0 mL of negative control plasma into the tubes labeled **NC** and **PC**.
- E. Pipette a 1.0-mL aliquot of the test sample into the tube labeled **TS₁**.
- F. Vortex-mix the contents of each tube for 5 - 10 seconds.

XVI. SOLID PHASE EXTRACTION

- A. Place a stopcock for the test sample and each control sample 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 a 13 x 100-mm conical tube labeled **SB₁** in the collection rack position that will be used for the corresponding test sample. Collect 2 mL of the elution solvent. Remove the tube and save until step XVI.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 conical 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, **S₁**, by pipetting 150 μL of aminorex working standard solution (0.10 $\text{ng}/\mu\text{L}$) and 150 μL of *cis*-4-methylaminorex working internal standard solution (0.1 $\text{ng}/\mu\text{L}$) into a 13 x 100-mm conical tube labeled **S₁**.
- O. Pipette 50 μL of *cis*-4-methylaminorex working internal standard solution (0.1 $\text{ng}/\mu\text{L}$) into the solution contained in tube **SB**. Vortex-mix the contents of the tube for 3 – 5 seconds.
- P. Evaporate the contents of each tube to dryness under nitrogen in a water bath at 40 ± 5 °C.
- Q. Prepare the system wash tubes by labeling two 13 x 100-mm conical tubes **SYS₁** and **SYS₂**.
- R. Add 30 μL acetonitrile to the tube labeled **S₁** and add 10 μL of acetonitrile to each of the remaining tubes. Vortex-mix the contents of each tube for 5 – 10 seconds.
- S. Add 150 μL 0.1% aqueous formic acid to the tube labeled **S₁** and add 50 μL of 0.1% aqueous formic acid to each of the remaining tubes. Vortex-mix the contents of each tube for 5 – 10 seconds.
- T. 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.
- U. Cap and submit the vials for LCMS analysis.

XVII. 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)

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- b. Column type: Metasil™ Polaris C18-A, (cat. no. 2000-100X030, Varian Consumable Products)
- 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: 45 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

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- d. Octapole 1 voltage: 11V
- e. Octapole 2 voltage: 2.0V
- 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 of aminorex from horse plasma.

Run #	Vial	Method	Sample
1	1	AMINOREX.M	PC
2-3	2-3	AMINOREX.M	SYS₁, SYS₂
4	4	AMINOREX.M	NC
5	5	AMINOREX.M	TS ₁
6	6	AMINOREX.M	SB ₁
7	7	AMINOREX.M	S ₁

C. Evaluation of Mass Spectral Data for Aminorex

1. Obtain the extracted ion chromatograms and retention times for the qualifying ions for the analyte and internal standard listed in Table 3 for the test sample extract, each control sample 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 the test sample extract, each control sample extract, 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 the test sample extract, each control sample extract, and the standard.

Table 3. Qualifying ions for LCMS analysis for aminorex in horse plasma; 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)
Aminorex	163 → 120 , <u>163</u>
<i>cis</i> -4-Methylaminorex (ISTD)	177 → 134 , <u>177</u>

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4. Measure the signal-to-noise ratio of the less abundant qualifying ion as indicated in Table 3 at the retention time of aminorex for the test sample extract.
 5. 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 each control sample extract, the test sample extract, and the solvent blank from the corresponding data files.
- D. Criteria for Identification of Aminorex from Plasma Extracts
1. The retention times of the qualifying ions for aminorex in the test sample extract and the positive control extract 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 the test sample extract, the control extracts, and the solvent blank 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 the test sample extract and the positive control extract 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.
 4. The relative ion area ratios of the qualifying ions for the internal standard in the test sample extract, the control extracts, and the solvent blank 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

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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 the test sample and from the standard must have essentially the same fragmentation patterns. Mass fragments not observed in the standard must not exceed 10% relative abundance in the test sample extract.

XVIII. 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 positive control sample, **PC**, does not contain aminorex according to the identification criteria specified for test samples as described in XVII.D.
- B. The test sample extract, control sample extracts, or the solvent blank do not contain the internal standard as evidenced by the presence of the characteristic ions within the expected retention time window.
- C. The solvent blank or the negative control sample, **NC**, contain aminorex as evidenced by the presence of the characteristic ions within the expected retention time window.
- D. There is an interfering substance in the test sample. Refer to Section XX.
INTERFERING SUBSTANCES.

XIX. 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 identification criteria described in XVII.D.
- B. The signal-to-noise ratio of the less abundant qualifying ion for aminorex in the

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test sample extract is greater than 10.

- C. Mass fragments not observed in the standard do not exceed 10% relative abundance in the test sample extract.
- D. The presence of aminorex in the corresponding urine sample has been demonstrated by a second analytical methodology, e.g., gas chromatography/mass spectrometry.

XX. 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.

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XXII. RESPONSIBLE PERSONS

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

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