

**For Testing Integrity Program**

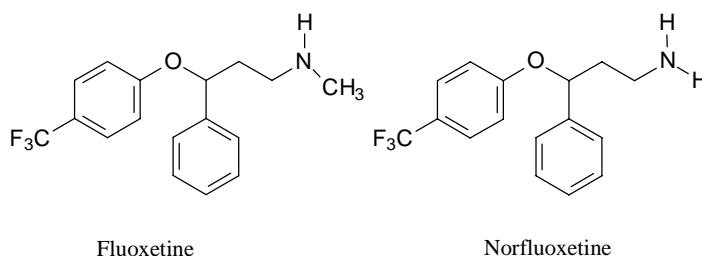
**IDENTIFICATION AND DETERMINATION OF FLUOXETINE AND NORFLUOXETINE FROM HORSE URINE**

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**I. INTRODUCTION**

Fluoxetine (Figure 1) is a member of a new class of antidepressant drugs that inhibit the release or inhibit the actions of serotonin in the central nervous system. Fluoxetine is used as an anti-depressant drug and to treat obsessive-compulsive disorders and bulimia. Due to its potential to affect the performance of a horse in a race the ARCI has classified fluoxetine in class 2.

Fluoxetine is extensively metabolized in persons to norfluoxetine and a number of other oxidative metabolites. Results of preliminary studies in horses indicate that norfluoxetine is also a major metabolite of fluoxetine in horses.



**Figure 1 Fluoxetine and norfluoxetine**

**II. SCOPE**

This standard operating procedure specifies a method to identify and determine fluoxetine and norfluoxetine from extracts of horse urine by gas chromatography / mass spectrometry. The lower limits of quantitation of this method for determination of fluoxetine and norfluoxetine in horse urine are approximately 20 ng/mL and 10 ng/mL, respectively.

**III. PRINCIPLE OF METHOD**

Fluoxetine, norfluoxetine, and the internal standard are extracted from basified urine by liquid-liquid extraction into dichloromethane-isopropanol after treatment of the urine with

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$\beta$ -glucuronidase. The analytes and the internal standard fluoxetine- $d_5$  are then isolated from other substances in the extraction mixture by solid phase extraction. After evaporation of the elution solvent, the residue is treated with heptafluorobutyric anhydride in the presence of pyridine to form the heptafluorobutyramide derivatives (Figure 2).

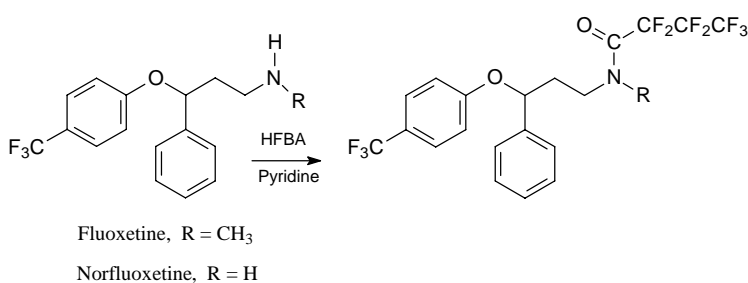


Figure 2 Derivatization of fluoxetine and norfluoxetine with HFBA

The derivatized analytes are extracted from the reaction mixture to remove excess reagent. The residue remaining after evaporation of the extraction solvent is dissolved in ethyl acetate and subjected to gas chromatographic / mass spectral analysis in the selected ion monitoring mode of operation under electron-impact ionization conditions. The concentrations of fluoxetine and norfluoxetine are determined by linear regression analysis of the peak area ratios of calibrators.

IV. REAGENTS

A. Water

Use double distilled water in any reagent or procedure requiring the use of water.

B. 1 M Acetate Buffer (pH 5.0)

1. Reagents

- a. Sodium acetate, anhydrous, reagent grade
- b. Concentrated glacial acetic acid, reagent grade
- c. Water

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2. Procedure
    - a. Dissolve 246 g of sodium acetate in approximately 1000 mL of water.
    - b. Add 99 mL of concentrated glacial acetic acid.
    - c. Dilute to 3000 mL with water. Mix.
  3. Storage Requirements
    - a. Store at approximately 4°C in a glass container.
    - b. Discard 1 year after preparation.
- C.  $\exists$ -Glucuronidase reagent (2500 units/mL in sodium acetate buffer)
1. Reagents
    - a.  $\exists$ -Glucuronidase from *Patella vulgata* (cat. no. G-8132, Sigma Chemical Co., St. Louis, MO 63178) or equivalent
    - b. 1 M acetate buffer (pH 5.0)
  2. Procedure
    - a. Dilute the contents of two vials (1,000,000 units per vial) of  $\exists$ -glucuronidase reagent to 800 mL with 1 M acetate buffer. Mix.
  3. Storage Requirements
    - a. Store at approximately 4 °C in a glass container.
    - b. Discard 2 months after preparation.
- D. 1.0 M carbonate buffer containing bromthymol blue
1. Reagents
    - a. Water
    - b. Bromthymol blue (cat. no. B-8630, Sigma Chemical Co., St. Louis, MO 63178)
    - c. Disodium carbonate, reagent grade
  2. Procedure

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- a. Dissolve 106 g of disodium carbonate and 200 mg of bromthymol blue in sufficient water to produce 1000 mL of solution. Mix.
3. Storage Requirements
  - a. Store at room temperature in a glass container.
  - b. Discard 1 year after preparation.
- E. Dichloromethane-isopropanol (3:1; v/v)
  1. Reagents
    - a. Dichloromethane, reagent grade
    - b. Isopropanol, reagent grade
  2. Procedure
    - a. 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.
- F. Glacial acetic acid, reagent grade
- G. 10 N Potassium hydroxide reagent

**NOTE: Preparation of this reagent generates heat.**

1. Reagents
  - a. Potassium hydroxide pellets, reagent grade
  - b. Water
2. Procedure
  - a. **Prepare under a fume hood.**



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3. Storage Requirements
  - a. Store at room temperature in a glass container.
  - b. Discard 1 year after preparation.
  
- J. 6 N Hydrochloric acid reagent
  1. Reagents
    - a. Concentrated hydrochloric acid, reagent grade
    - b. Water
  2. Procedure
    - a. **Prepare under a fume hood.**
    - b. Add one volume of concentrated hydrochloric acid to an equal volume of water. Mix.
  3. Storage Requirements
    - a. Store at room temperature in a glass container.
    - b. Discard 1 year after preparation.
  
- K. Diluted ammonium hydroxide reagent (1:1; v/v)
  1. Reagents
    - a. Concentrated ammonium hydroxide, reagent grade
    - b. Water
  2. Procedure
    - a. **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 glass container.

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- L. Methanol (B&J Brand™ cat. no. 230-4, B&J, Muskegon, MI 49442-6184 or equivalent)
- M. 1 N Acetic acid reagent
  - 1. Reagents
    - a. Glacial acetic acid, reagent grade
    - b. Water
  - 2. Procedure
    - a. Add 17.6 mL of glacial acetic acid to sufficient water to produce 300 mL of solution. Mix.
  - 3. Storage Requirements
    - a. Store at room temperature in a glass container.
    - b. Discard 1 year after preparation.
- N. Dichloromethane-isopropanol-ammonium hydroxide (78:20:2; v/v/v) reagent
  - 1. Reagents
    - a. Dichloromethane, (B&J ACS/HPLC Certified, cat. no. AH300-4, B&J or equivalent)
    - b. Isopropanol, reagent grade
    - c. Concentrated ammonium hydroxide, reagent grade
  - 2. Procedure
    - a. **Prepare under a fume hood.**
    - b. Combine 78 mL of dichloromethane and 20 mL of isopropanol.
    - c. Add 2 mL of ammonium hydroxide. Mix.
  - 3. Storage Requirements
    - a. Prepare the reagent fresh daily.
    - b. Store at room temperature in a glass container.

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- O. Heptafluorobutyric anhydride (HFBA, cat. no. 18118, Alltech Associates Inc., Deerfield, IL 60015-1899)
- P. 50% Pyridine in toluene reagent
  - 1. Reagents
    - a. Pyridine (B&J Brand™ cat. no. 331, B&J or equivalent)
    - b. Toluene (B&J Brand™ cat. no. 347-4, B&J or equivalent)
  - 2. Procedure
    - a. **Prepare under a fume hood. Wear Barrier® laminated film gloves (Ansell Edmont, cat. no. 2-100, Coshocton, OH 43812) when handling pyridine and the pyridine solution.**
    - b. Combine 1 mL of pyridine and 1 mL of toluene. Mix.
  - 3. Storage Requirements
    - a. Prepare the reagent fresh daily.
    - b. Store at room temperature in a glass container.
- Q. Dichloromethane (cat. no. AH300-4, B&J or equivalent)
- R. Saturated sodium borate reagent
  - 1. Reagents
    - a. Sodium tetraborate decahydrate, granular
    - 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 appearance of an insoluble residue.
  - 3. Storage Requirements
    - a. Store at room temperature in a glass container.

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b. Discard 1 year after preparation.

S. Ethyl acetate (no.100-4, B&J or equivalent)

T. Nitrogen gas

**V. SUPPLIES**

A. 16 × 125 mm glass culture tubes with caps.

B. 13 × 100 mm glass round bottom screw cap tubes with caps.

C. 13 × 100 mm glass conical centrifuge tubes with caps.

D. Solid phase extraction columns (cat. no. ZSDAU020, United Chemical Technologies, Bristol, PA 19007).

E. Tissue paper wipers (e.g. Kimwipes®).

F. 100- $\mu$ L autosampler vials with 11 mm crimp caps (cat. no. 9301-0977, 5282-1210 Hewlett-Packard Co., Palo Alto, CA 94304 or equivalent).

G. Glass pasteur pipettes, disposable.

**VI. APPARATUS**

A. Pipettes with disposable tips.

**Note: use the following positive displacement pipettes to pipette the standard solutions and working standard solutions.**

1. 1 - 10 :L positive displacement pipette (microman, cat. no. m10, Rainin Instrument Co., Inc., Woburn, MA 01888-4026).

2. 10 - 100 :L positive displacement pipette (microman, cat. no. m100, Rainin Instrument Co., Inc.).

3. 200 - 1000 :L positive displacement pipette (microman, cat. no. m1000, Rainin Instrument Co., Inc.).

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4. 10 – 100  $\mu$ L adjustable volume pipettor (Eppendorf 4810, Brinkmann Instruments Inc, Westbury, NY 11590).
  5. 100 - 1000  $\mu$ L adjustable volume pipettor (pipet-plus, cat. no. R-1000, Rainin Instrument Co., Inc. or Eppendorf 4810, Brinkmann Instruments Inc.).
  6. 1 - 10 mL electronic pipettor (edp plus, Rainin Instrument Co., Inc.).
- B. Vortex mixer (American Scientific Products, McGaw Park, IL 60085 or equivalent).
  - C. pH meter (Accumet model 610, Fisher Scientific Co., Pittsburgh, PA 15219 or equivalent).
  - D. Incubator (Lab-Line Instruments Inc., Melrose Park, IL 60160 or equivalent).
  - E. Rotorack (Glas-Col® Apparatus Co., Terre Haute, IN 47802 or equivalent).
  - F. Centrifuge capable of centrifuging 13 x 100 mm round-bottom test tubes at 2000 - 3000 rpm (model HN-SII, Damon/IC division, Needham Heights, MA 02194 or equivalent).
  - G. Evaporator capable of evaporating solvent from 13 x 100 mm round-bottom conical test tubes and 13 x 100 conical test tubes without heat under nitrogen (The Meyer N-Evap, Organomation Assoc. Inc., South Berlin, MA 01549 or equivalent).
  - H. Varian Vac Elut SPS 24™ solid phase extraction apparatus (cat. no. 1223-4022, Varian Sample Preparation Products, Harbor City, CA 90710).
  - I. Heating block capable of heating 13 x 100 mm round-bottom test tubes at 90 °C for 30 minutes (Lab-Line Instruments Inc, Melrose Park, IL 60160 or equivalent).

**VII. TEST SUBSTANCE**

The test substance for this procedure is horse urine or diluted horse urine.

**VIII. VOLUME REQUIRED**

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Use 1 mL or appropriate dilution of each test sample in duplicate. Refer to section XIII.D for preparation of appropriate dilutions of test samples.

**IX. STANDARD SOLUTIONS**

- A. Fluoxetine working standard solution in methanol – 1.00 µg/µL
  - 1. Reagents
    - a. Fluoxetine (USP Reference Standards, Rockville, MD 20852)
    - b. Methanol (B&J Brand™ cat. no. 230-4, B&J, Muskegon, MI 49442-6184 or equivalent)
  - 2. Procedure
    - a. Dissolve 11.2 mg of fluoxetine hydrochloride in sufficient methanol to produce 10.0 mL of solution. Prepare a second 1.00 µg/µL standard solution.
    - b. Store the standard solutions at approximately 4 °C.
- B. Norfluoxetine HCl standard solution - 1 mg/mL (gift from PETRL).
- C. Fluoxetine-*d*<sub>5</sub> standard solution - 1.0 µg/µL in methanol (cat. no.82-602-02-0, Isotech, Miamisburg, OH 45342).

**X. WORKING STANDARD SOLUTIONS**

**Note:** Independently prepare two working standard solutions each of fluoxetine and norfluoxetine for this analysis. Use one working standard solution to prepare the calibrators and standard and the other one to prepare the positive control samples.

- A. Fluoxetine working standard solution in methanol - 10.0 ng/µL
  - 1. Reagents
    - a. Fluoxetine standard solution in methanol – 1.00 µg/µL
    - b. Methanol (B&J Brand™ cat. no. 230-4 or equivalent)
  - 2. Procedure

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- a. Dilute 100  $\mu$ L of fluoxetine standard solution to 10.0 mL with methanol in a volumetric flask.
  - b. Prepare a second 10.0-ng/ $\mu$ L fluoxetine working standard solution by repeating step 2.a.
  - c. Store the working standard solutions at approximately 4 °C.
- B. Fluoxetine working standard solution in methanol - 1.00 ng/ $\mu$ L
1. Reagents
    - a. Fluoxetine working standard solution in methanol – 10.0 ng/ $\mu$ L
    - b. Methanol (B&J Brand™ cat. no. 230-4 or equivalent)
  2. Procedure
    - a. Dilute 1.00 mL of 10.0 ng/ $\mu$ L-fluoxetine working standard solution to 10.0 mL with methanol in a volumetric flask.
    - b. Prepare a second 1.00-ng/ $\mu$ L fluoxetine working standard solution by repeating step 2.a.
    - c. Store the working standard solutions at approximately 4 °C.
- C. Norfluoxetine working standard solution in methanol - 10.0 ng/ $\mu$ L
1. Reagents
    - a. Norfluoxetine HCl standard solution - 1 mg/mL (gift from PETRL).
    - b. Methanol (B&J Brand™ cat. no. 230-4 or equivalent)
  2. Procedure
    - a. Dilute 50  $\mu$ L of norfluoxetine standard solution to 5.0 mL with methanol in a volumetric flask.
    - b. Prepare a second norfluoxetine working standard solution by repeating step 2.a.
    - c. Store the working standard solutions at approximately 4 °C.
- D. Norfluoxetine working standard solution in methanol - 1.00 ng/ $\mu$ L
1. Reagents

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- a. Norfluoxetine HCl working standard solution - 10.0 ng/:L
- b. Methanol (B&J Brand™ cat. no. 230-4 or equivalent)

2. Procedure

- a. Dilute 1.00 mL of 10.0 ng/:L-norfluoxetine standard solution to 10.0 mL with methanol in a volumetric flask.
- b. Prepare a second 1.00-ng/:L norfluoxetine working standard solution by repeating step 2.a.
- c. Store the working standard solutions at approximately 4 ° C.

E. Fluoxetine-*d*<sub>5</sub> working standard solution in methanol - 10.0 ng/:L

1. Reagents

- a. Fluoxetine-*d*<sub>5</sub> standard solution - 1.0 mg/mL in methanol (cat. no.82-602-02-0, Isotech, Miamisburg, OH 45342).
- b. Methanol (B&J Brand™ cat. no. 230-4 or equivalent)

2. Procedure

- a. Dilute 100 µL of the standard solution to a 10.0 mL with methanol in a volumetric flask.
- b. Store the working standard solution at approximately 0 °C.

**XI. CONTROL SAMPLES**

- A. Horse urine sample negative for fluoxetine and norfluoxetine.
- B. Fluoxetine/Norfluoxetine positive control samples containing 100 ng/mL each

**Note:** prepare the positive control samples using working standard solutions prepared independently from those used to prepare the calibrators.

1. Add 40.0 :L each of 10.0 ng/:L-fluoxetine and 10.0 ng/:L-norfluoxetine working standard solutions to a labeled 16 × 125 mm test tube. Evaporate the methanol to dryness under nitrogen without heat.
2. Add 4.0 mL of negative horse urine to the tube and vortex-mix for 3 - 5 seconds.

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3. Store at approximately  $-20^{\circ}\text{C}$ .

**XII. SAMPLE REQUIREMENTS FOR ANALYSIS**

The following samples and standards are required for each analysis:

1. Calibrators designated **C<sub>1</sub>**, **C<sub>2</sub>**, **C<sub>3</sub>**, **C<sub>4</sub>**, and **C<sub>5</sub>**; prepare calibrators at concentrations of 20, 50, 100, 200, and 500 ng/mL for fluoxetine and 10, 20, 50, 100 and 200 ng/mL of norfluoxetine from negative control horse urine and the working standard solutions of fluoxetine and norfluoxetine.
2. System Blanks designated **SYS<sub>1</sub>** and **SYS<sub>2</sub>**; prepare system blanks from ethyl acetate.
3. Negative control sample designated **NC**; prepare negative control sample from negative control urine.
4. Test sample(s) designated **TS<sub>1a...TS<sub>nb</sub></sub>** where n is the total number of test samples; a and b are designations for sample replicates.
5. Solvent blank(s) designated **SB<sub>1a...SB<sub>nb</sub></sub>** where n is the total number of test samples; a and b are designations for sample replicates.
6. Positive control samples designated **PC<sub>a</sub>**, **PC<sub>b</sub>**, and **PC<sub>c</sub>**; a, b, and c are designations for sample replicates.
7. Standard mixture designated **S<sub>1</sub>**.

**XIII. CALIBRATOR AND SAMPLE PREPARATION**

- A. Pipette 5.0  $\mu\text{L}$  of fluoxetine-*d*<sub>5</sub> working standard solution into each appropriately labeled 16  $\times$  125 mm test tubes except those labeled **SYS<sub>1</sub>**, **SYS<sub>2</sub>**, **SB<sub>1a...SB<sub>nb</sub></sub>**, and **S<sub>1</sub>**.

**NOTE:** Prepare **S<sub>1</sub>** during step XVII.N, and **SYS<sub>1</sub>** and **SYS<sub>2</sub>** during step XIX.H.

- B. Pipette 20.0, 50.0, and 100.0  $\mu\text{L}$  of 1.00-ng/:L fluoxetine working standard solution and 10.0, 20.0, and 50.0  $\mu\text{L}$  of 1.00-ng/:L norfluoxetine working standard solution into the tubes labeled **C<sub>1</sub>**, **C<sub>2</sub>**, and **C<sub>3</sub>**, respectively; pipette 20.0 and 50.0  $\mu\text{L}$  of 10.0-ng/:L fluoxetine working standard solution and 10.0 and 20.0  $\mu\text{L}$  of

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10.0-ng/;L norfluoxetine working standard solution into the tubes labeled **C<sub>4</sub>** and **C<sub>5</sub>**, respectively. Evaporate the methanol to dryness without heat. See Table 1.

**Note:** Use different working standard solutions from those used to prepare the positive control samples.

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

TUBE NO.	Volume of Fluoxetine and Norfluoxetine Working Standard Solutions, :L	Volume of Fluoxetine -d <sub>5</sub> Working Standard Solution, :L	Fluoxetine and Norfluoxetine Injected into GC/MS, ng	Fluoxetine-d <sub>5</sub> Injected into GC/MS, ng	Equivalent to Fluoxetine and Norfluoxetine in the Urine, ng/mL	Equivalent to Fluoxetine-d <sub>5</sub> in the Urine, ng/mL
<b>C<sub>1</sub></b>	20.0/10.0 <sup>1</sup>	5.0	0.2/0.1	0.5	20/10	50
<b>C<sub>2</sub></b>	50.0/20.0 <sup>1</sup>	5.0	0.5/0.2	0.5	50/20	50
<b>C<sub>3</sub></b>	100.0/50.0 <sup>1</sup>	5.0	1.0/0.5	0.5	100/50	50
<b>C<sub>4</sub></b>	20.0/10.0 <sup>2</sup>	5.0	2.0/1.0	0.5	200/100	50
<b>C<sub>5</sub></b>	50.0/20.0 <sup>2</sup>	5.0	5.0/2.0	0.5	500/200	50
<b>SYS<sub>1-2</sub></b>	0	0	0	0	na	na
<b>NC</b>	0	5.0	0	0.5	0	50
<b>TS<sub>1a-1b</sub></b>	0	5.0	unknown	0.5	unknown	50
<b>SB<sub>1a-1b</sub></b>	0	0	0	0	na	na
<b>PC<sub>a-c</sub></b>	40* <sup>2</sup>	5.0	1.0/1.0	0.5	100/100	50
<b>S<sub>1</sub></b>	20.0/20.0 <sup>2</sup>	5.0	2.0/2.0	0.5	na	na

na = not applicable \*per 4 mL total urine volume

<sup>1</sup> 1.00 ng/μL fluoxetine and norfluoxetine working standard solutions; <sup>2</sup> 10.0 ng/μL fluoxetine and norfluoxetine working standard solutions;

- C. Pipette 1.0 mL of negative control urine into the tubes labeled **NC**, **C<sub>1</sub>**, **C<sub>2</sub>**, **C<sub>3</sub>**, **C<sub>4</sub>**, and **C<sub>5</sub>**.
- D. Pipette duplicate 1.0 mL-aliquots of the test sample into the tubes labeled **TS<sub>1a</sub>** and **TS<sub>1b</sub>**, if the estimated concentration of fluoxetine is between 20 and 500 ng/mL and that of norfluoxetine is between 10 and 200 ng/mL. If the estimated concentration of fluoxetine is greater than 500 ng/mL or that of norfluoxetine is greater than 200 ng/mL, prepare appropriate dilution(s) of an aliquot of the test sample with negative control urine so that the estimated concentration of

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fluoxetine is between 20 and 500 ng/mL and that of norfluoxetine is between 10 and 200 ng/mL. Pipette 1.0 mL of the diluted sample into the tubes labeled **TS<sub>1a</sub>** and **TS<sub>1b</sub>**. Repeat this process for each test sample.

- E. Pipette 1.0 mL of the positive control sample into each of the tubes labeled **PC<sub>a</sub>**, **PC<sub>b</sub>**, and **PC<sub>c</sub>**.
- F. Pipette 1.0 mL of water into the tubes labeled **SB<sub>1a</sub>**..**SB<sub>nb</sub>**.
- G. Add 4.0 mL of water to each tube.
- H. Vortex-mix the contents of each tube for 5 - 10 seconds.

**XIV. ENZYME HYDROLYSIS OF CONJUGATES**

- A. Pipette 2 mL of  $\beta$ -glucuronidase reagent into each tube.
- B. Vortex-mix the contents of each tube for 5 -10 seconds.
- C. Adjust the contents of each tube to pH 4.5 - 5.5 with 1 *N* hydrochloric acid, 6 *N* hydrochloric acid or dilute ammonium hydroxide solution.
- D. Place the tubes in an incubator at approximately 65 °C for approximately 2 hours.
- E. Remove the tubes from the incubator and allow them to cool to room temperature.

**XV. EXTRACTION OF ANALYTES**

- A. Pipette 2 mL of 1.0 *M* sodium carbonate buffer containing bromthymol blue into each tube.
- B. Pipette 5 mL of dichloromethane - isopropanol (3:1; v/v) into each tube.
- C. Cap the tubes; mix the contents of each tube 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 each top layer by aspiration.

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- F. Decant each bottom layer to a new, appropriately labeled round bottom 13 x 100 mm tube.
- G. Add 25 :L of glacial acetic acid to each tube.
- H. Vortex-mix the contents of each tube for 3 - 5 seconds.
- I. Evaporate the contents of each tube to dryness under nitrogen without heat.

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

- A. Pipette 2 mL of 0.1 *M* potassium phosphate buffer (pH 6.0) into each tube.
- B. Adjust the contents of each tube to pH 5.5 - 6.5, if necessary, with 1*N* hydrochloric acid, 6 *N* hydrochloric acid or dilute ammonium hydroxide reagent.

**XVII. SOLID PHASE EXTRACTION PROCEDURE**

- A. Place a stopcock for each test sample, calibrator, and control sample on the stainless steel delivery tips of the Vac-Elut device. 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 dichloromethane-isopropanol-ammonium hydroxide (78:20:2; v/v/v) reagent.
- C. Remove the Vac-Elut lid and wipe the collection needles with a paper tissue.
- D. Place 13 × 100 mm round bottom tubes labeled **SB<sub>1a</sub>..SB<sub>nb</sub>** in the collection rack position that will be used for the corresponding test sample. Collect 3 mL of dichloromethane-isopropanol-ammonium hydroxide (78:20:2; v/v/v) reagent. Remove the tubes and set aside until step XVII.O.
- E. Place a solid phase column on each stopcock.
- F. Condition each solid phase column by applying low vacuum (1 - 5 mm of Hg) and successively eluting to waste 3 mL of methanol, 3 mL of water, and 1 mL of 0.1 *M* potassium phosphate buffer (pH 6.0). Stop the flow as soon as each reagent reaches the top of the sorbent bed.

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- G. Decant the test samples, calibrators, and control samples into the column reservoirs and adjust the flow through each column to 1 - 2 mL/minute.
- H. Rinse each column with 3 mL of water.
- I. Rinse each column with 1 mL of 1 *N* acetic acid reagent.
- J. Rinse each column with 3 mL of methanol.
- K. Dry the columns under full vacuum for 5 minutes.
- L. Place labeled 13 x 100 mm round bottom tubes into position under the corresponding collection needles. Verify that the needles are positioned into the tubes.
- M. Elute to collect with 3 mL of dichloromethane-isopropanol-ammonium hydroxide (78:20:2; v/v/v) reagent.
- N. Prepare the standard mixture by pipetting 20 :L each of 10 ng/ $\mu$ L-fluoxetine and 10 ng/ $\mu$ L-norfluoxetine working standard solutions and 5.0 :L of fluoxetine-*d*<sub>5</sub> working standard solution into a 13 × 100 mm round bottom tube labeled **S**<sub>1</sub>.
- O. Pipette 5.0 :L of fluoxetine-*d*<sub>5</sub> working standard solution into the solution contained in tubes **SB**<sub>1</sub>...**SB**<sub>n</sub>.
- P. Evaporate the contents of each tube to dryness under nitrogen in an evaporator without heat.

**XVIII. DERIVATIZATION PROCEDURE**

- A. Add 100 :L of HFBA to each tube from step XVII.P.
- B. Add 20 :L of 50% pyridine in toluene reagent to each tube.
- C. Cap and vortex mix the contents of each tube for 3 - 5 seconds.
- D. Place all tubes in a heating block at approximately 90 °C for approximately 30 minutes.

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- E. Remove the tubes from the heating block and allow them to cool to room temperature.
- F. Evaporate the contents of each tube to dryness, not exceeding a total of 5 minutes, under nitrogen in an evaporator without heat.

**XIX. POST DERIVATIZATION PROCEDURE**

- A. Add 3 mL of dichloromethane and 2 mL of saturated sodium borate reagent solution to each tube from step XVIII.F.
- B. Cap and mix by end-over-end rotation for 5 - 10 minutes at 5 – 20 rpm.
- C. Centrifuge at 2000 - 3000 rpm for 5 - 10 minutes.
- D. Aspirate the aqueous (top) phase from each tube.
- E. Decant each organic (bottom) layer into an appropriately labeled conical tube.
- F. Evaporate the contents of each tube to dryness under nitrogen in an evaporator without heat. Remove each tube from the evaporator as soon as evaporation of the liquid is complete. Excessive evaporation times may result in loss of analytes.
- G. Prepare the system blank tubes by labeling two 13 × 100 mm conical tubes **SYS<sub>1</sub>** and **SYS<sub>2</sub>**.
- H. Add 100 :L of ethyl acetate to each tube from steps XIX.F and XIX.G.
- I. Cap and vortex-mix the contents of each tube for 3 - 5 seconds.
- J. Submit the samples for GC/MS analysis.

**XX. GAS CHROMATOGRAPHIC/MASS SPECTRAL IDENTIFICATION OF FLUOXETINE AND NORFLUOXETINE**

- A. Gas Chromatographic and Mass Spectrometer Operating Parameters
  - 1. Instrumentation: Hewlett-Packard GC/MSD  
equipped with HP MS  
Chemstation operating software

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(MS-DOS and MS-Windows)

2. GC column:
  - a. column type: DB-1 or DB-5MS (J&W Scientific)
  - b. column length: 15 meters
  - c. column i.d.: 0.25 mm
  - d. film thickness: 0.25 :m
  
3. Carrier gas:
  - a. type: Helium ultra-high purity (99.999%)
  - b. flow rate: 1.0 mL/min
  - c. column pressure: 4 psi
  
4. Injection:
  - a. type: splitless
  - b. injection volume: 1 :L
  - c. divert off time: 0.8 minute
  
5. Autosampler:
  - a. type: model 7673 (Hewlett-Packard)
  - b. sample washes: 0
  - c. sample pumps: 4
  - d. viscosity delay: 0 seconds
  - e. solvent washes: 6
  
6. Temperatures:
  - a. injector: 280 EC
  - b. oven temperature program: 70 EC (hold for 0.5 minute after injection) and then increase at 15 EC/minute to 280 EC (return to 70 EC)
  - c. interface: 280 EC
  
7. Source:

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- a. pressure: 5 - 8 x 10<sup>-6</sup> Torr
- b. temperature: determined by the interface
  
- 8. Ionization:
  - a. electron-impact
  
- 9. Programs:
  - a. name: 1SIFLUOX.M - selected ion monitoring program
    - (1) Start time: 9.6 minutes
    - (2) Dwell time: 20 msec
    - (3) EMV offset: 0 volts
    - (4) Ions monitored: 117.30, 122.30, 226.30, 330.30, 344.30, and 349.30 amu
  - b. Name: FSFLUOX.M - full scan program
    - (1) Start time: 9.6 minutes
    - (2) Low mass: 50
    - (3) High mass: 400
    - (4) Threshold: 10

**B. Procedure**

1. Transfer the contents of each tube to a 100- $\mu$ L autosampler vial using a new pasteur pipette for each transfer.
2. Perform GC/MS analysis in the order specified in Table 3.

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Table 2. Order of analysis including vial numbers, sample names, methods, and sample descriptions for identification and determination of fluoxetine and norfluoxetine.

Vial Number	Sample Name	Method	Description
1	CC	1SIFLUOX.M	Check Sample
2	C <sub>1</sub>	1SIFLUOX.M	Calibrator 1
3	C <sub>2</sub>	1SIFLUOX.M	Calibrator 2
4	C <sub>3</sub>	1SIFLUOX.M	Calibrator 3
5	C <sub>4</sub>	1SIFLUOX.M	Calibrator 4
6	C <sub>5</sub>	1SIFLUOX.M	Calibrator 5
7	SYS <sub>1</sub>	1SIFLUOX.M	System Blank 1
8	SYS <sub>2</sub>	1SIFLUOX.M	System Blank 2
9	NC	1SIFLUOX.M	Negative Control
9	NC	FSFLUOX.M	Negative Control
10	TS <sub>1a</sub>	1SIFLUOX.M	Test Sample 1
10	TS <sub>1a</sub>	FSFLUOX.M	Test Sample 1
11	SB <sub>1a</sub>	1SIFLUOX.M	Solvent Blank 1
11	SB <sub>1a</sub>	FSFLUOX.M	Solvent Blank 1
12	TS <sub>1b</sub>	1SIFLUOX.M	Test Sample 2
12	TS <sub>1b</sub>	FSFLUOX.M	Test Sample 2
13	SB <sub>1b</sub>	1SIFLUOX.M	Solvent Blank 2
13	SB <sub>1b</sub>	FSFLUOX.M	Solvent Blank 2
14	PC <sub>a</sub>	1SIFLUOX.M	Positive Control 1
15	PC <sub>a</sub>	1SIFLUOX.M	Positive Control 2
16	PC <sub>c</sub>	1SIFLUOX.M	Positive Control 3
17	S <sub>1</sub>	1SIFLUOX.M	Standard Mix
17	S <sub>1</sub>	FSFLUOX.M	Standard Mix

C. Evaluation of Mass Spectral Data for Fluoxetine and Norfluoxetine

1. Obtain the total ion chromatogram (TIC), the integrated ion areas (A<sub>117</sub>, A<sub>174</sub>, A<sub>344</sub>, A<sub>226</sub>, A<sub>330</sub>, and A<sub>349</sub>), and retention times for each of the qualifying ions listed in Table 3 for each test sample, solvent blank, calibrator, and control sample.
2. Calculate the relative ion area ratios for each of the analytes by dividing the qualifying ion area by the ion area of the most abundant ion as indicated in Table 3 for each test sample, calibrator, positive control, and standard.

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Table 3. Qualifying and quantifying ions for analysis for fluoxetine and norfluoxetine; the most abundant qualifying ions are indicated in **bold** type and the least abundant qualifying ions are underlined.

Analyte	Qualifying Ions (amu)	Quantifying Ions (amu)
Fluoxetine-HFB	<b>117</b> , <u>174</u> , 344	344
Norfluoxetine-HFB	<b>117</b> , <u>226</u> , 330	330
Fluoxetine- <i>d</i> <sub>5</sub> -HFB	<b>122</b>	349

3. Calculate the peak area ratio for each analyte by dividing the area of the respective quantifying ion (*i.e.*,  $A_{\text{ion}(m/z)}$ ) at the retention time of the derivative by the area of the quantifying ion from the internal standard (*i.e.*,  $A_{349}$ ) at the retention time of the internal standard derivative.
4. Measure the signal-to-noise ratio for the least abundant qualifying ion for the fluoxetine and norfluoxetine derivatives in both test sample extracts. Least abundant qualifying ions are listed and underlined in Table 3.
5. Print the full scan spectra of the fluoxetine and norfluoxetine derivatives from the standard full scan data file. Print the full scan spectra found at the retention times of the fluoxetine and norfluoxetine derivatives from the negative control sample extract, each test sample extract, and solvent blank from the respective full scan data files.

D. Criteria for Identification of Fluoxetine and Norfluoxetine from Urine Extracts

1. The retention times of the qualifying ions for the target analyte in each replicate of the test sample must be within  $\nabla$  0.05 minutes of the retention times of the qualifying ions from the corresponding standard.
2. The relative ion area ratios of the qualifying ions for the analytes in each replicate of the test sample must be within  $\nabla$  20% of the values of the same ions from the corresponding standard.
3. The chromatographic peak shape must be approximately Gaussian, with a narrow base, with baseline separation from neighboring peaks, and with

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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.
4. The full scan spectra for the fluoxetine and norfluoxetine derivatives from each test sample extract and from the standard have essentially the same fragmentation pattern and the retention times are within 0.05 minutes (*i.e.*, the retention times of the fluoxetine derivatives from the test sample extracts and the standard are within 0.05 minutes).

E. Determination of the Concentration Fluoxetine and Norfluoxetine in Urine

1. Plot the peak area ratios of the quantifying ions for fluoxetine and norfluoxetine versus the respective concentration in the calibrators. Perform linear regression analysis on these data to obtain the slope, intercept, and correlation coefficient of each of the standard curves.
2. Calculate the concentrations of fluoxetine and norfluoxetine in each test sample or diluted test sample and the control samples from the peak area ratios of the quantifying ions and the slope and intercept of the standard curve.
3. Calculate the concentrations of fluoxetine and norfluoxetine in the diluted test sample from the calculated concentration and the dilution factor used:

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

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4. Calculate the mean concentration of fluoxetine and norfluoxetine for each test sample and the positive control sample.

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

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

Report a test sample positive for fluoxetine (or norfluoxetine) when all of the following criteria are met:

- A. The signal-to-noise ratio of the ion at  $m/z$  174 for fluoxetine-HFB (or  $m/z$  226 for norfluoxetine-HFB) from both replicates of the test sample (or diluted test sample) is greater than 3.
- B. The negative control sample extracts and the system and solvent blanks do not contain fluoxetine or norfluoxetine.
- C. Each standard curve has a correlation coefficient greater than 0.98.
- D. The mean fluoxetine (and norfluoxetine) concentrations in the positive control samples meet the criteria for acceptability as defined in the Quality Manual.

**XXII. INTERFERING SUBSTANCES**

It is possible that a large amount of one or more substances in the extract could react with the derivatizing agent and thereby interfere with derivatization of the analytes. This would be evident by a reduction in the response of the internal standard. Appropriate corrective actions would include modification of the extraction procedure to reduce the amount of interfering substances and the use of a larger amount of HFBA to achieve complete derivatization.

**XXIII. PROCEDURAL NOTES**

Water, alcohols, and other compounds containing active hydrogen atoms react rapidly with HFBA causing it to decompose and form HFB derivatives. This may interfere with the formation of fluoxetine and norfluoxetine derivatives and may result in the occurrence of interfering peaks in the chromatogram. Therefore, it is important to reduce or eliminate the presence of such compounds, particularly water, by following the procedure as described and verifying that the extracts are dry before adding the derivatization reagents.

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During the derivatization procedure an oily residue instead of a white precipitate may form during the evaporation step. The oily residue does not solidify with increased evaporation time and continuing with the procedure for no more than 5 minutes of total evaporation time has resulted in acceptable data.

During validation of this method, it was determined that chromatographic peak shape was unacceptable when toluene was used as the injection solvent. Acceptable peak shape was obtained when ethyl acetate was used as the injection solvent.

**XXIV. REFERENCES**

- A. United Chemical Technologies Clean Screen Extraction Columns Applications Manual, Bristol, PA, 19007 circa 1991.
- B. Ryan, M., Todi, F., and Mendonca, M. Analysis of fluoxetine in equine urine and blood by pre-column derivatisation with dansyl chloride and liquid chromatography. *Proc. 11th Int. Conf. Racing Anal. Vet.* **11**:158-162, 1996.

**XXV. RESPONSIBLE PERSONS**

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

**Prepared by:**

Shelley Smith and Gary Roberts

**Approved by:**

Lucille Kaminski - QAO      Date

Richard Sams - Director      Date