IDENTIFICATION AND DETERMINATION GABAPENTIN FROM HORSE URINE SAMPLES BY LIQUID CHROMATOGRAPHY - MASS SPECTROMETRY

Developed

by

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Gabapentin is a both an analgesic and anticonvulsant drug (Neurontin). It is not a Drug Enforcement Administration controlled substance and although approved for epilepsy treatment most of sales seem to be related to other conditions, e.g. for neurogenic pain management. It has been classified by the Association of Racing Commissioners International (ARCI) as a class 4 drug in horses.

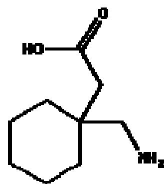

Figure 1. Structure of Gabapentin.

I. SCOPE

This standard operating procedure describes procedures to be used to identify and quantify Gabapentin (Figure 1) from horse urine. The limit of quantitation of this method for determination of Gabapentin in urine is approximately 5 ng/mL.

II. PRINCIPLE OF METHOD

Gabapentin is isolated from horse urine by solid-phase extraction using a positive pressure manifold and IST HCX-3 SPE cartridges. The method, in brief, consists of loading buffered specimens (containing internal standards), rinsing with water, acidifying with dilute acetic acid solution (to lock the basic drugs onto the cation exchange mode of cartridge), then rinsing with methanol, drying column, and finally collection of the Basic drug fraction by elution with 78% methylene chloride + 20 % propanol with 2% Ammonium Hydoxide. The extracts are evaporated then redissolved and determinations are made by liquid chromatography / mass spectrometry. The concentration of Gabapentin is determined by the internal standard method using the peak area ratio and linear regression analysis mathod. Phenylpropanolamine may be used as an internal standard.

III. REAGENTS

1. Methanol, Optima grade (cat. no. A454, Thermo Fisher Scientific or equivalent)
2. Glacial Acetic Acid, HPLC Grade (cat. no. A35-500, Thermo Fisher Scientific or equivalent)
3. Deionized (DI) Nanopure Water (for operation of Nanopure System)
4. Ammonium Hydroxide Solution, reagent grade (Thermo Fisher Scientific or equivalent)

5. 2 N Hydrochloric acid Solution (cat. no. SA431-500, Thermo Fisher Scientific or equivalent)
6. Monobasic sodium phosphate (NaH_2PO_4) reagent grade or better (Thermo Fisher Scientific or equivalent)
7. Formic Acid (EM Science 98% pure, WR Scientific)

IV. SOLUTIONS

A. Elution solvent: Methylene Chloride:2-Propanol:Ammonium Hydroxide (78:20:2; v/v/v)

1. Reagents
   a) Methylene Chloride, Optima grade (cat. no. D-151, Fisher Scientific, Pittsburgh, PA or equivalent)
   b) 2-Propanol, Optima grade (cat. no. A4664, Fisher Scientific or equivalent)
   c) Concentrated Ammonium Hydroxide, reagent grade
2. Procedure
   a) Prepare under a fume hood.
   b) To a 100 mL graduate cylinder, add 20 mL of propanol, then 2 mL of ammonium hydroxide. Mix.
   c) Dilute to 100 mL with Methylene Chloride
3. Storage Requirements
   a) Prepare the reagent fresh daily.
   b) Store at room temperature in a glass container.

B. 1.6 M Acetate Buffer, pH 5.0 (1 L)

1. Reagents
   a) sodium acetate trihydrate
   b) acetic acid
   c) DI water
2. Procedure
   a) Place 136 g sodium acetate trihydrate in a 1 L flask. Add 33 mL acetic acid. Dilute to volume (1 L) with DI water. Check pH (5.0 ± 0.1).
3. Storage Requirements
   a) Store at room temperature in a glass container. Discard after 3 months or if contamination evident

V. MATERIALS

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

B. 12 × 75 mm glass culture tubes with snap caps.

C. Pipettes and tips.

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

1. 0.1 - 10 µL adjustable volume pipette (Eppendorf 2100, Brinkmann Instruments Inc., Westbury, NY 11590-0207).
2. 2.0 - 20 ul adjustable volume pipette (Eppendorf 2000, Brinkmann Instruments Inc.).
3. 10 - 100 µL adjustable volume pipette (Eppendorf 2000, Brinkmann Instruments Inc.).
4. 20 - 200 µL adjustable volume pipette (Eppendorf 2000, Brinkmann Instruments Inc.).
5. 100 - 1000 µL adjustable volume pipette (Eppendorf 2000, Brinkmann Instruments Inc.).
6. 500 - 5000 µL adjustable volume pipette (Eppendorf 2100, Brinkmann Instruments Inc.).

D. Vortex Mixer, Multi-Pulse (Glas-Col® Apparatus Co., Terre Haute, IN 47802

E. Bransonic Ultrasonic Water Bath, 5510 (Thermo Fisher Scientific).

F. pH Meter (Corning 445, Thermo Fisher Scientific or equivalent)

G. pH Paper, full range kit (Thermo Fisher Scientific or equivalent)

H. Centrifuge (Sorvall Super T21, Kendro Laboratory Products, Newtown, CT -6470 or equivalent).

I. Cerex 48-place solid phase extraction apparatus (Cera Inc. Baldwin Park, CA).

J. Solid Phase Extraction Columns, IST HCX-3 SPE cartridges, 3cc/130mg (cat No.905-0013-13, Argonaut Technologies, Foster City, CA or equivalent)

K. Evaporator (TurboVap, Zymark, Cambridge, MA or equivalent).

L. Glass pasteur pipettes, disposable.

M. Bottle Top Dispensers (0.4-2 ml, 1-3 mL, 1-10 ml, 2-10 ml) Brinkmann Dispensette (Brinkmann Instruments Inc., Westbury, NY 11590-0207).

N. Volumetric Flasks, 2, 5, 10 50, 100, 1000 mL (Class A Pyrex, Thermo Fisher Scientific or equivalent).

O. Mixing Cylinder, 100 mL and 1 L (Pyrex, Thermo Scientific or equivalent).

VI. TEST SUBSTANCE

horse urine

VII. VOLUME REQUIRED

1 mL or appropriate dilution. Use duplicate 1 mL aliquots of undiluted test sample if the estimated concentration of Gabapentin in the test sample is between 5.0 and 200 ng/mL. If the estimated concentration Gabapentin is greater than 200 ng/mL, prepare an appropriate dilution of an aliquot of the test sample with water. Repeat this process for each test sample. Use this same dilution with water to prepare the negative control urine for the standard curve.

VIII. WORKING STANDARD SOLUTIONS

A. Gabapentin standard solution in methanol - 1.0 ng/µL

1. Gabapentin (1.0 mg/mL solution in methanol, cat no. G-007, Cerilliant or equivalent)

2. Prepare a 1.0 ng/µL solution by diluting 10 µL of the 1.0 mg/mLGabapentin stock solution to 10 mL in a class A volumetric flask with methanol.

3. Store at approximately < 0°C when not in use, discard after use

Prepare two separate working standard solutions; use one solution for the preparation of the calibrators and the standard mixture and the other solution for the preparation of the control samples.

B. Pseudoephdrine internal standard working solution in methanol - 10.0 ng/µL

1. Pseudoephdrine (1.0 mg/mL solution in methanol, cat no. P-035, Cerilliant or equivalent)

2. Prepare a 1.00 µg/µL stock solution of Pseudoephdrine as per SOP B001. Store at < 0°C.

3. Prepare a 10.0 ng/µL solution of Pseudoephdrine by diluting 10 µL of the 1.00 µg/µL Pseudoephdrine standard solution to 1.0 mL with methanol in a volumetric flask. Store < 0°C, discard after 1 month.

CONTROL SAMPLES

A. Negative Control Sample - Horse urine demonstrated by analysis to contain no detectable Gabapentin.

1. Dilute the negative control sample with water to the same extent as the test sample (see section VIII. VOLUME REQUIRED).

2. Store control urine at <0 °C when not in use.

B. Positive Control Sample - Horse urine supplemented with 100 ng/mL Gabapentin.

1. Prepare as described below in Table 1. Use tubes labeled PC_a, PC_b, and PC_c and the Gabapentin working standard solution that was not used for preparation of calibrators.

IX. SAMPLE REQUIREMENTS FOR ANALYSIS

Prepare the following samples and standards for each analysis:

1. Calibrators designated $\mathbf{C_1}$, $\mathbf{C_2}$, $\mathbf{C_3}$, $\mathbf{C_4}$, $\mathbf{C_5}$, and $\mathbf{C_6}$; prepare calibrators at concentrations described in Table 1 from negative control urine or diluted negative control urine and Gabapentin working standard solution.

2. System washes designated $\mathbf{SYS_1}$ and $\mathbf{SYS_2}$; prepare system washes from LC mobile Phase.

3. Negative control sample designated **NC**; prepare negative control sample from negative control urine or diluted negative control urine.

4. Test sample(s) designated $\mathbf{TS_{1a}...TS_{nb}}$ where n is the total number of test samples; a and b are designations for sample replicates.

5. Solvent blank(s) designated $\mathbf{SB_{1a}...SB_{nb}}$ where n is the total number of test samples; a and b are designations for sample replicates.

6. Positive control samples designated $\mathbf{PC_a}$, $\mathbf{PC_b}$, and $\mathbf{PC_c}$ where a, b, and c are designations for sample replicates.

7. Standard mixture designated $\mathbf{S_1}$.

X. CALIBRATOR AND SAMPLE PREPARATION

A. Pipette 10 µL of Pseudoephdrine internal standard working solution (10 ng/µL) into each labeled 12 × 75 mm test tube except those labeled $\mathbf{SYS_1, SYS_2, SB_{1a}..SB_{nb}}$ and $\mathbf{S_1}$.

B. Pipette Gabapentin working standard solution (1 ng/µL) into the calibrator tubes labeled $\mathbf{C_1}$, $\mathbf{C_2}$, $\mathbf{C_3}$, $\mathbf{C_4}$, $\mathbf{C_5}$ and $\mathbf{C_6}$ as described in Table 1.

C. Pipette Gabapentin working standard solution No. 2 (1 ng/µL) into the calibrator tubes labeled $\mathbf{PC_a, PC_b,}$ and $\mathbf{PC_c}$.

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

TUBE NO.	Volume of Gabapentin Working Standard Solution, µL	Volume of Pseudoephdrine Working Standard Solution, µL	Equivalent to Gabapentin in the Urine, ng/mL	Equivalent to Pseudoephdrine in the Urine, ng/mL
C_1	5	10	5	100
C_2	10	10	10	100
C_3	20	10	20	100
C_4	40	10	40	100
C_5	100	10	100	100
C_6	200	10	200	100
SYS_{1-2}	0	0	na	na
NC	0	10	0	100
TS_{1a-1b}	0	10	unknown	100
SB_{1a-1b}	0	0	na	na
PC_{a-c}	100	10	100	100
S_1	100	10	na	na

na = not applicable

D. Evaporate solvent in all tubes using TurboVap concentrator and then pipette 1 mL of negative control urine or diluted negative control urine into the tubes labeled **NC**, $\mathbf{PC_{a-c}}$**,** $\mathbf{C_1}$, $\mathbf{C_2}$, $\mathbf{C_3}$, $\mathbf{C_4}$, $\mathbf{C_5}$ and $\mathbf{C_6}$.

E. Pipette duplicate 1 mL aliquots of the test sample or diluted test sample into the tubes labeled $\mathbf{TS_{1a}}$ and $\mathbf{TS_{1b}}$. Repeat this process for each test sample.

F. Pipette 1 mL of water into each tube labeled $\mathbf{SB_{1a}}$...$\mathbf{SB_{nb}}$.

G. Vortex mix the contents of each tube for 30 seconds.

H. Sonicate all sample tubes (e.g. in Bransonic water bath @ 65°C for 30 min) before SPE to break up components that may clog SPE cartridges. Also, centrifuge samples (e.g. 3,100 rpm x 1 min), if necessary.

XI. SOLID-PHASE EXTRACTION PROCEDURE

A. Adjust pH and Centrifuge Specimens

1. Adjust the pH of urine specimens to 5 ± 0.1 by adding 2 mL of pH 5 1.6 M Acetate Buffer to each specimen.

B. Condition the IST HCX-3 SPE cartridges: 3cc/135mg SPE Column

1. Place the solid phase columns on the manifold rack, and condition each solid phase column by applying a small amount of pressure (1-5 psi) and successively eluting to waste 3 mL of methanol and 3 mL of water.

C. 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.

D. Rinse each column with 3 mL of water.

E. Acidify each column with 3 mL of 1 M acetic acid solution.

F. Rinse each column with 3 mL of methanol.

G. Dry the columns under full vacuum for 2 minutes at 20 psi.

H. Collect Drug Fraction

1. Place a rack with new, labeled 12 × 75 mm tubes into the SPE manifold collection rack. Pass through the column and collect 3 mL of the following solvent (flow rate 1 to 2 mL/min):

   a) Methylene Chloride:2-Propanol:Ammonium Hydroxide (78:20:2; v/v/v)

Let soak for 1 minute before using pressure - if needed.

2. After all solvent has passed through the column expel any eluent remaining in the column into collection vial by applying low-pressure nitrogen.

I. Prepare the standard mixture by adding 100 μL of Gabapentin and 10 μL of Pseudoephdrine working solutions to a 12 X 75 mm tube labeled $\mathbf{S_1}$.

J. Evaporate the contents of each tube to dryness under nitrogen in a TurboVap concentrator bath at 40 ± 5 °C.

XII. REDISSOLVING PROCEDURE

A. Prepare the system wash tubes by labeling two autosampler vials with inserts **SYS_1** and **SYS_2**.

B. Add 150 µL of Sample Re-dissolving Solvent (2% Acetonitrile in water with 0.2% formic acid) to each tube.

C. Cap and vortex mix the contents of each tube for 30 seconds.

D. Transfer to pre-labeled autosampler vials with inserts and submit for LC/MS analysis.

XIII. LIQUID CHROMATOGRAPHIC AND MASS SPECTROMETER OPERATING PARAMETERS

A. Instrumentation:

1. Thermo Mass Spectrometer and Agilent Technologies Model 1100 HPLC pump, autosampler, column compartment, and degasser. Xcalibur™ software (Thermo Scientific Inc., San Jose, CA) used for system control and data processing on a MS Windows platform, or equivalent.

2. LC column:
   a) type: Advanced Chromatography Technology ACE C18 (cat no. ACE 3 C18-A1554, Mac Mod Analytical Technologies or equivalent)
   b) length: 100 mm
   c) i.d.: 2.1 mm
   d) particle size: 3 µm

3. Mobile Phase

Time (min)	% ACN +0.2% Formic Acid	% Water +0.2% Formic Acid
0.0	2	98
0.5	2	98
5.0	40	60
6.0	90	10
7.0	90	10
7.01	2	98
12.0	2	98

a) Solvents: HPLC Grade, Burdick & Jackson
b) flow rate: 0.4 mL/min

4. Injection volume: 40 µL

5. Ionization and Detection
Positive Ion Electrospray with Full Scan LC-MS/MS Detection of the pseudomolecular ions of Gabapentin and Pseudoephdrine -IS
a) Maximum Ion Inject time: 100 msec
b) Number of Microscans: 1
c) Gabapentin MS/MS/MS Transitions followed: 172.1 amu (isolation width 1.5, collision energy 35 %) → 154.1 amu (isolation width 1.5, collision energy 36%) → 137 and 119 from Full Scan MS^3 (90 - 180 amu)
d) Pseudoephdrine -IS MS/MS Transitions followed: 133.1 amu (isolation width 1.5, collision energy 35 %) → 117 from Full Scan MS^2 (100 - 160 amu)

6. Program:
Name: Gabapentin_Confirm

B. Procedure

1. Transfer the contents of each tube to an autosampler vial with a ~150-µL insert using a new disposable pipette for each tube.

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

Table 2. Run number, vial number, acquisition method, and sample designation for LC-MS analysis for identification and determination of Gabapentin from urine.

Run #	Vial	Method	Sample
1-6	1-6	Gabapentin_Confirm	$\mathbf{C_1, C_2, C_3, C_4, C_5, C_6}$
7-8	7-8	Gabapentin_Confirm	$\mathbf{SYS_1, SYS_2}$
9	9	Gabapentin_Confirm	**NC**
10	10	Gabapentin_Confirm	$\mathbf{TS_{1a}}$
11	11	Gabapentin_Confirm	$\mathbf{SB_{1a}}$
12	12	Gabapentin_Confirm	$\mathbf{TS_{1b}}$
13	13	Gabapentin_Confirm	$\mathbf{SB_{1b}}$
14-16	14-16	Gabapentin_Confirm	$\mathbf{PC_a}$, $\mathbf{PC_b}$, and $\mathbf{PC_c}$
17	17	Gabapentin_Confirm	$\mathbf{S_1}$

C. Evaluation of Mass Spectral Data for Gabapentin

1. Obtain the total ion chromatogram (TIC), the integrated ion areas and retention times for the qualifying and quantifying ions of Gabapentin and Pseudoephdrine internal standard listed in Table 3 for each calibrator, test sample, positive control sample, and the standard.

2. Calculate the relative ion area ratio for the Gabapentin by dividing the qualifying ion area by the ion area of the most abundant qualifying ion as indicated in Table 3 for each replicate of the test sample and the standard.

3. Calculate the relative ion area ratio for the Pseudoephdrine by dividing the area of the qualifying ion by the area of the respective quantifying ion as indicated in Table 3 for each replicate of the test sample and the standard.

Table 3. Qualifying and quantifying ions for analysis of Gabapentin in extracts of horse urine; 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)
Gabapentin	119, **137,** 109	119 + 137
Pseudoephdrine	**117**, 133	133

4. If an internal standard curve is to be used Calculate the peak area ratio for the Gabapentin by dividing $A_{119+137}$ at the retention time of the Gabapentin by A_{133} at the retention time of the Pseudoephdrine for each calibrator, test sample and positive control sample. If an external standard curve is to be used plot the $A_{119+137}$ of Gabapentin against the concentration for calibrators using linear regression.

5. Print the full scan spectrum at the retention time of Gabapentin for each replicate of each test sample.

6. Print the full scan spectrum of the analyte from the standard full scan data file. At the retention time of the analyte, select and print the full scan spectrum from the negative control sample extract, test sample extracts, and solvent blanks from the corresponding data files.

D. Criteria for Identification of Gabapentin from Urine Extracts

1. The retention times of the qualifying ions in the test sample must be within ± 0.1 minutes of the retention time of the same ions from the corresponding standard.

2. The relative ion area ratios for the Gabapentin from each replicate of the test sample must be within ± 25% of the values of the same ions from the corresponding standard. The most abundant ion must be the quantifying ion indicated in Table 3.

3. The chromatographic peak shape must be approximately Gaussian, with a narrow base, with baseline separation from neighboring peaks, and with little evidence of tailing. The following criteria will define an acceptable peak:

   a) The width of the peak at its base should be less than 0.20 minutes.

   b) The peak should appear to be Gaussian, *i.e*, symmetrical about the vertical mid-line.

   c) There should be no interfering peaks. A neighboring peak is considered to be interfering if the height from the baseline to the lowest part of the valley between the peaks is greater than 10% of the height of the peak of interest.

   d) There is no significant peak tailing. Unacceptable peak tailing is defined as the condition in which the ratio of *b* to *a* is greater than 1.1 at 11% 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.

4. The full scan spectra for the Gabapentin from each test sample extract and from the standard must have essentially the same fragmentation patterns and the retention times must be within 0.1 minutes (*i.e.*, the retention times of the Gabapentin from the test sample extracts and the standard must be within 0.1 minutes).

E. Determination of the Concentration of Gabapentin in Urine

1. Plot the peak area ratios of the quantifying ions for each calibrator versus the concentration of the analyte in the calibrator. Perform linear regression analysis on these data to obtain the slope, intercept, and correlation coefficient of the standard curve.

2. Calculate the concentration of the analyte in each test sample and positive control sample from the peak area ratios of the quantifying ions and the slope and intercept of the corresponding standard curve.

3. Determine the average concentration for each test sample and positive control sample.

Average concentration=1/2 (concentration $\mathbf{TS}_{1a}$ + concentration $\mathbf{TS}_{1b}$)

XIV. CRITERIA FOR REPEATING THE ANALYSIS

Repeat the analysis of the test sample if any of the following conditions apply:

A. The peak area of any test sample replicate is greater than the peak area of calibrator C_6. Repeat the analysis after diluting the urine sample with water as described in Section VIII of this Standard Operating Procedure.

B. The negative control sample or the solvent blanks contain Gabapentin as evidenced by the presence of the characteristic ions within the expected retention time window.

C. The standard curve for Gabapentin has a correlation coefficient less than 0.98.

D. The Pseudoephdrine ions are not detectable within the expected retention time window for any of the sample replicates.

E. The average concentration of the positive control sample replicates is not within ± 20% of the nominal concentration.

XV. CRITERIA FOR REPORTING A SAMPLE POSITIVE FOR GABAPENTIN

A. Report a test sample as positive per this standard operating procedure for Gabapentin if all of the following criteria are met:

1. The test sample contains Gabapentin according to the criteria described in XV.D.

2. The average concentration of Gabapentin in the test sample is greater than 5 ng/mL.

3. The signal-to-noise ratio of the least abundant qualifying ion for Gabapentin in each replicate of the test sample is greater than 3.

XVI. RESPONSIBLE PERSONS

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

Prepared by: University of California, Davis

Approved by:

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Scott Stanley – University of California   Date

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Richard Sams, TIP Chairman   Date