CONFIRMATION OF GABAPENTIN BY LIQUID CHROMATOGRAPHY- MASS SPECTROMETRY

35.1 METHOD

This test method may be used to confirm the presence of gabapentin (GABA) in biological specimens. GABA and internal standard (GABA-d_{10}) are isolated from biological matrices by protein precipitation and solid-phase extraction (SPE). The extracts are injected into a high performance liquid chromatograph (HPLC) coupled to a mass spectrometer (MS) detector equipped with an atmospheric pressure electrospray ionization source.

35.2 SPECIMENS

The specimen volume is 0.5 mL. Specimens include, but are not limited to, whole blood, serum, plasma, urine, and tissue homogenate. Dilutions of specimens may be analyzed at the Forensic Scientist's discretion.

NOTE: Method validation established that matrix-matching of the full calibration curve and all positive control levels is not required for quantitation in liver (tissue) homogenate specimens (see 35.4.3.4). Matrix-matching of the full calibration curve and all positive control levels is required for quantitation in serum/plasma specimens (see 35.4.2 and 35.4.3).

35.3 REAGENTS, MATERIALS AND EQUIPMENT

35.3.1 REAGENTS

NOTE: Laboratory general-use DI H_2O and reagent grade organic solvents are used in reagent preparation, unless otherwise specified.

- Acetonitrile (ACN), reagent grade and LC-MS grade
- Ammonium hydroxide (NH_4OH), concentrated
- Certified blank blood and/or other biological matrices
- DI H_2O, laboratory general-use and LC-MS grade H_2O (or equivalent from a high-purity filtration system)
- Elution solvent

  To 98 mL MeOH, add 2 mL concentrated NH_4OH and mix. Store the elution solvent in glass flask/bottle at room temperature and use on date of preparation only.
- Formic acid (concentrated)
- 0.1% Formic acid in LC-MS grade H_2O

  Add 1 mL of concentrated formic acid to 800 mL LC-MS grade H_2O in a 1 L flask and mix. Dilute to 1 L with LC-MS grade H_2O and mix. Store the acid in a glass bottle at room temperature for up to one year.

NOTE: Filtration prior to use is not required for 0.1% formic acid unless DI H_2O must be used in place of LC-MS grade H_2O.

- Hydrochloric acid (HCl), concentrated
- 0.1M HCl

  To 400 mL DI H_2O, add 4.2 mL concentrated HCl. Dilute to 500 mL with DI H_2O and mix. Store the acid in a glass bottle at room temperature for up to 6 months.

- Methanol (MeOH), reagent grade and HPLC grade

  NOTE: Adjustments to final volumes of prepared reagents are permitted as long as the proportions are maintained.

35.3.2 MATERIALS

- Disposable extraction tubes (16 x 100 mm recommended) and screw-cap or centrifuge tubes with closures
- Extraction column: United Chemical Technologies' Clean Screen SPE cartridge (CSDAU206 200mg/6mL), or equivalent
- HPLC Column, Agilent Zorbax Eclipse Plus C18, 4.6 x 75 mm, dp = 3.5 µM, or equivalent
- Laboratory glassware (graduated cylinders, flasks)
- Polypropylene autosampler vials with integrated inserts and caps

35.3.3 EQUIPMENT

- Agilent HPLC (1100/1200 series, or equivalent)
- Agilent MS with API-ES source (SL model, or equivalent)
- Calibrated, adjustable piston pipettes and verified, adjustable repeater-pipette with disposable pipette tips
- General-use equipment (centrifuge, evaporator, vacuum manifold, vortex mixer)

35.4 STANDARDS, CALIBRATORS AND CONTROLS

35.4.1 STANDARDS

- Working standard (WS): 0.1 mg/mL
- Working control standard (QC): 0.1 mg/mL
- Stock internal standard: 100 µg/mL (0.1 mg/mL)

NOTE: Certified reference material GABA-d_{10} stock standard is used directly in sample preparation.

35.4.2 CALIBRATORS

Calibrators are prepared in certified blank blood at the time of analysis, as detailed in 35.5 SAMPLE PREPARATION. Quantitation in serum/plasma

specimens requires that a calibration curve be prepared in blank matrix. If testing only serum/plasma specimens, a blood calibration curve is not required.

35.4.3 CONTROLS

35.4.3.1 At least one negative blood control and two positive blood controls are included in the batch, prepared as described in 35.5. For quantitative analysis of serum/plasma specimens only, blood controls are not required.

35.4.3.2 Controls (positive or negative) must make up at least 10% of the extracted batch (based on number of case specimen samples), with case specimens bracketed by positive controls. When the batch contains more than 20 specimens, a third positive control (low or high) must be extracted and analyzed mid-run.

35.4.3.3 For qualitative analysis of any alternate matrices, one negative control and one positive control must be included for each alternate matrix type tested in the batch.

35.4.3.4 For quantitative analysis of liver (tissue) homogenate specimens, matrix-matching of the full calibration curve and all positive controls is not required. One negative control and one positive control must be included in the batch. Positive controls in both blood and/or tissue homogenate serve to bracket tissue case specimens and apply towards 10% of the batch.

35.4.3.5 For quantitative analysis of serum/plasma specimens, matrix-matching of the full calibration curve and all positive controls (to meet 10% and bracket specimens in that matrix) is required.

35.5 SAMPLE PREPARATION

35.5.1 Label a clean extraction tube for each member of the test batch. (i.e., calibrator, control, case sample).

35.5.2 Using a calibrated pipette, add 0.5 mL of certified blank blood into each of the six calibrator tubes, the positive control tubes and the negative control tube(s).

35.5.3 Prepare a 1:10 dilution of the working standard. (0.01 mg/mL)

a. Using a calibrated pipette, combine 100 µL of the working standard with 900 µL of ACN or MeOH in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after calibrator preparation.

35.5.4 Using a calibrated pipette, spike the calibrators according to the following table, using the working standard and the prepared dilution.

Calibrator Description	Volume (µL) Added	Standard Concentration	Dilution of WS (or WS)
Calibrator 1 – 1.0 mg/L	50	0.01 mg/mL	1:10
Calibrator 2 – 2.0 mg/L	100	0.01 mg/mL	1:10
Calibrator 3 - 5.0 mg/L	25	0.1 mg/mL	WS
Calibrator 4 - 10 mg/L	50	0.1 mg/mL	WS
Calibrator 5 - 15 mg/L	75	0.1 mg/mL	WS
Calibrator 6 - 20 mg/L	100	0.1 mg/mL	WS

35.5.5 Using a calibrated pipette, spike the positive controls according to the following table, using the working control standard.

Control Description	Volume (µL) Added	Standard Concentration	Dilution of QC (or QC)
Control 1 – 3.0 mg/L	15	0.1 mg/mL	QC
Control 2 - 16 mg/L	80	0.1 mg/mL	QC

35.5.6 Using a calibrated pipette, sample 0.5 mL of each case specimen into its respective tube.

35.5.7 Using a calibrated pipette or verified repeater-pipette, add 20 µL of the stock internal standard solution to each tube. Final concentration of the internal standard is 4 mg/L.

35.5.8 Add 1 mL ACN to each tube.

35.5.9 Cap the tubes and briefly vortex mix. Centrifuge the tubes for 5 minutes at 3000 rpm (recommended for 16 x 100 mm tubes).

35.5.10 Transfer the supernatant to a new tube and add 2 mL 0.1M HCl. Vortex briefly.

35.5.11 Place new, labeled SPE columns into the vacuum manifold.

35.5.12 Condition the SPE columns by passing each of the following solvents completely through under force of gravity.

a. 3 mL MeOH
b. 1 mL 0.1M HCl

Do not let columns dry out between each conditioning step.

35.5.13 Transfer the contents of each tube to its respective SPE column and allow them to flow through under force of gravity. (Moderate, positive pressure or vacuum may be applied if the flow is insufficient.)

35.5.14 Wash the SPE columns by passing 1 mL 0.1M HCl completely through under force of gravity. (Moderate, positive pressure or vacuum may be applied if the flow is insufficient.)

35.5.15 Dry the columns for 5 minutes under vacuum.

35.5.16 Place clean, labeled centrifuge tubes in the collection rack underneath their corresponding SPE columns.

35.5.17 Pass 2 mL of elution solvent through each SPE column and collect the extracts.

35.5.18 Transfer the tubes to the evaporator and evaporate the extracts to dryness at 50°C.

35.5.19 Reconstitute the extracts with the addition of 100 µL HPLC grade MeOH to each tube. Briefly vortex mix the tubes. Centrifuge the tubes for 2 minutes at 2000 rpm (recommended) to collect the extracts at the bottom of the tubes.

35.5.20 Transfer the extracts to labeled polypropylene autosampler vials with integrated inserts and cap.

35.6 INSTRUMENTAL PARAMETERS/DATA ANALYSIS

- Acquisition method – GABA (instrumental parameters in Appendix A)
- Calibration curve – linear, 1/a weighting factor
- Updating calibrator (retention times ±3%, ion ratios ±20%) – Cal 4
- Result comparisons –

  Cal 1: acceptable range ±25% (0.75 – 1.25 mg/L), truncated to two decimal places

  Cals 2-6, Ctrls 1-2: acceptable range ±20%, truncated to one decimal places

35.7 REPORTING

Results are reported in units of milligrams per liter (mg/L), truncated to two significant figures.

35.1 METHOD PERFORMANCE

- Limit of detection: 0.1 mg/L
- Lower limit of quantification: 1.0 mg/L
- Dynamic range: 1.0 – 20 mg/L
- Upper limit of quantitation: 20 mg/L
- Upper limit of linearity: 30 mg/L

35.2 REFERENCES

- Katie Harris and Dawn Sklerov, in-house method development
- A.F. Lehner, J. Stewart, A. Dafalla, K.J. Ely, A.L. Connerly, C.N. Joe, H. ElkHoly, K. Thompson, T. Tobin and L. Dirikolu, *Gabapentin in Horses: Validation of an Analytical Method for Gabapentin Quantitation*, J Anal Tox. **30**: 555-565 (2007).

APPENDIX A
INSTRUMENTAL PARAMETERS

LIQUID CHROMATOGRAPH

Gradient Elution	
Flow rate	0.8 mL/min
Solvent A	0.1% Formic acid in LC-MS grade H_2O
Solvent B	ACN (LC-MS grade)
Initial composition	90% A, 10% B
0 – 5.0 min	% B increased to 50%
Hold time	5.0 min (50% B)
Post time	5.0 min
Column temp	40°C
Autosampler	
Injection volume	1.0 µL
Injection flush-port	Active
Flush-port time/volume	15 sec
Flush-port solvent	ACN (LC-MS grade)

MASS SPECTROMETER

Scan type	(+) SIM	Nebulizer gas	Nitrogen
Ion mode	ESI	Nebulizer pressure	40 psi
Peak width	0.08 min	Drying gas	Nitrogen
EM Gain	1.0	Drying gas flow	13 L/min
		Drying gas temp	350 °C
		Capillary voltage	4 kV

Compound	Ions	Ion Ratios
Gabapentin-d_{10}	182, 164	164/182
Gabapentin	172, 137, 154	137/172, 154/172

LIST OF CHANGES

Revision Date	Description	Page Number
5/20/15	Method approved by Washington State Toxicologist. See DRA dated 5/11/15. Method released for use in evidentiary testing as of 5/20/15.	All
8/24/15	Note added to 35.6.1.2, 35.6.1.4 removed and amount of internal standard added in 35.7.8 changed to 20µL to reflect use of the GABA-d_{10} CRM directly in sample preparation. Nebulizer pressure changed to 40 psi in instrument parameters.	3-4, 6, 10
3/16/16	Added note regarding CRM expiration dates in 35.6.1.3. Added clarification to 35.6.3.2.c for use of same CRM in preparation of working standard and working control standard. Edited 35.12.1.1 to reflect that only two significant figures are used for reporting. Other minor edits throughout.	2-4, 8
6/12/17	Wording was added to section 35.4.3 regarding dilution and standard volume testing. Specified the use of calibrated pipettes for measurement of blank blood, specimens, and standards throughout section 35.7 SAMPLE PREPARATION. Edited section 35.10.2.2.d to indicate all positive controls must meet acceptability criteria for GABA to report quantitative results. Other minor edits throughout.	1-8
7/9/18	Removed policy, purpose and principle sections, summarizing under new section METHOD. Added specific wording regarding matrix-matching in 35.2 SPECIMENS, 35.4.2 CALIBRATORS and 35.4.3 CONTROLS. Edited STANDARDS section - this information is now included in the revised Standard Solution Preparation procedure. Specified use of LC-MS grade deionized water and acetonitrile in 35.3.1. Criteria for batch acceptance (calibrators, controls) and specimen acceptability criteria, and specific data analysis and reporting information are now included in the General Requirements for Chromatographic Test Method Batch Analysis and Acceptance. Changed run time (0-5 min) and % B (50) in liquid chromatograph instrumental parameters to match instrument acquisition method. Formatting and minor edits throughout.	All
6/15/20	Edited NOTE in 35.3.1; moved filtration information to NOTE in prep of 0.1% formic acid (no filtration required for prep with LC-MS grade H_2O). Changed references for "LC-MS grade DI H_2O" to "LC-MS grade H_2O." NOTE regarding specific grade of H_2O and solvents used was removed from 35.5 (covered in 35.3.1). Use of mid-run control was added in 35.4.3.2. Changed pipetted volumes in 35.5.3 from mL to µL. Updated retention time acceptability to ±3% in 35.6. Other minor edits throughout.	All