CONFIRMATION OF PREGABALIN AND GABAPENTIN BY LIQUID CHROMATOGRAPHY – TANDEM MASS SPECTROMETRY

48.1 METHOD

This test method may be used to confirm the presence of gabapentin (GABA) and pregabalin (PREG) in biological specimens. GABA, PREG, and internal standards (GABA-d_{10} and PREG-d_6) 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 tandem mass spectrometer (MS-MS) detector equipped with an atmospheric pressure electrospray ionization source.

48.2 SPECIMENS

The specimen volume is 0.2 mL. Specimens include, but are not limited to, whole blood, serum, plasma, urine, and tissue homogenate. For specimens analyzed at less than standard sample volume (e.g., limited specimen volume available), only qualitative results are reported. Specimens analyzed at standard sample volume with results >50 mg/L (ULOQ) are reported as >50 mg/L (see 48.7). Results from evaluation of dilution suitability in method validation for specimens analyzed at less than standard volume does not support quantitative reporting.

NOTE: Method validation established that matrix-matching of the full calibration curve and all positive control levels is not required for quantitation in serum specimens (see 48.4.3.4). Matrix-matching of the full calibration curve and all positive control levels is required for quantitation in tissue homogenate specimens (see 48.4.2 and 48.4.3).

48.3 REAGENTS, MATERIALS AND EQUIPMENT

48.3.1 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.

48.3.2 MATERIALS

- Disposable extraction tubes (12 x 75 and 16 x 100 mm) 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 150 mm, dp = 5 µM, or equivalent
- Laboratory glassware (graduated cylinders, flasks)
- Polypropylene autosampler vials with integrated inserts and caps

48.3.3 EQUIPMENT

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

48.4 STANDARDS, CALIBRATORS AND CONTROLS

48.4.1 STANDARDS

- Working standard (WS): 0.1 mg/mL
- Working control standard (QC): 0.1 mg/mL
- GABA-d_{10} Stock internal standard: 100 µg/mL (0.1 mg/mL)
- PREG-d_6 Stock internal standard: 100 µg/mL (0.1 mg/mL)

NOTE: Certified reference material GABA-d_{10} and PREG-d_6 stock standard is used directly in sample preparation.

48.4.2 CALIBRATORS

Calibrators are prepared in certified blank blood at the time of analysis, as detailed in 48.5 SAMPLE PREPARATION. Quantitation in tissue homogenate specimens requires that a calibration curve be prepared in blank matrix. If testing only tissue homogenate specimens, a blood calibration curve is not required.

48.4.3 CONTROLS

48.4.3.1 At least one negative blood control and three positive blood controls are included in the batch, prepared as described in 48.5.6. For quantitative analysis of tissue homogenate specimens only, tissue homogenate controls are required.

48.4.3.2 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, one of the three positive controls must be analyzed mid-run.

48.4.3.3 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.

48.4.3.4 For quantitative analysis of serum 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 serum serve to bracket tissue case specimens and apply towards 10% of the batch.

48.4.3.5 For quantitative analysis of tissue homogenate specimens, matrix-matching of all positive controls (to meet 10% and bracket specimens in that matrix) is required.

48.5 SAMPLE PREPARATION

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

48.5.2 Using a calibrated pipette, add 0.2 mL of certified blank blood into each of the six calibrator tubes, the positive control tubes and the negative control tube(s).

48.5.3 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.

48.5.4 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	20	0.01 mg/mL	1:10
Calibrator 2 – 2.0 mg/L	40	0.01 mg/mL	1:10
Calibrator 3 - 5.0 mg/L	100	0.01 mg/mL	1:10
Calibrator 4 - 10 mg/L	20	0.1 mg/mL	WS
Calibrator 5 - 25 mg/L	50	0.1 mg/mL	WS
Calibrator 6 - 50 mg/L	100	0.1 mg/mL	WS

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

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

48.5.6 Using a calibrated pipette, spike the positive controls according to the following table, using the working control standard and prepared dilution.

Control Description	Volume (µL) Added	Standard Concentration	Dilution of QC (or QC)
Control 1 – 3.0 mg/L	60	0.01 mg/mL	1:10
Control 2 - 15 mg/L	30	0.1 mg/mL	QC
Control 3 – 40 mg/L	80	0.1 mg/mL	QC

48.5.7 Using a calibrated pipette, sample 0.2 mL of each case specimen into its respective tube.

48.5.8 Using a calibrated pipette or verified repeater-pipette, add 20 µL of the GABA-D_{10} stock internal standard solution and 20 µL of the PREG-D_6 stock internal standard solution to each tube. Final concentration of the internal standard is 10 mg/L.

48.5.9 Add 1 mL ACN to each tube.

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

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

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

48.5.13 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.

48.5.14 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.)

48.5.15 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.)

48.5.16 Dry the columns for 5 minutes under vacuum.

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

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

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

48.5.20 Reconstitute the extracts with the addition of 200 µL 0.1% starting mobile phase composition (90:10 of 0.1% Formic acid in LC-MS grade H_2O and LC-MS grade ACN) 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.

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

48.6 INSTRUMENTAL PARAMETERS/DATA ANALYSIS

- Acquisition method – PREGAB (instrumental parameters in Appendix A)
- Calibration curve – linear, 1/a weighting factor
- Updating calibrator (retention times ±2%, 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 1-3: acceptable range ±20%, truncated to one decimal place

48.7 REPORTING

Results are reported in units of milligrams per liter (mg/L), truncated to two significant figures. Results greater than the ULOQ will be reported as >50 mg/L.

48.8 METHOD PERFORMANCE

- Limit of detection: 0.05 mg/L
- Lower limit of quantification: 1.0 mg/L
- Dynamic range: 1.0 – 50 mg/L
- Upper limit of quantitation: 50 mg/L
- Upper limit of linearity: 100 mg/L

48.9 REFERENCES

- K. Harris, D. Sklerov and A. Black, in-house method development
- *Confirmation of Gabapentin by Liquid Chromatography – Mass Spectrometry* (TCg12735, Rev. 5)
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- U. Mandal, A.K. Sarkar, K.V. Gowda, S. Agarwal, A. Bose, U. Bhaumik, D. Ghosh, T.K. Pal, *Determination of Pregabalin in Human Plasma Using LC-MS-MS*, Chromatographia **67**: 237-243 (2008).
- L. Nahar, A. Smith, R. Patel, R. Andrews, S. Paterson, *Validated Method for the Screening and Quantification of Baclofen, Gabapentin and Pregabalin in Human Post-Mortem Whole Blood Using Protein Precipitation and Liquid Chromatography – Tandem Mass Spectrometry*, J Anal Tox. **41**: 441-450 (2017).

APPENDIX A
INSTRUMENTAL PARAMETERS

LIQUID CHROMATOGRAPH

Gradient Elution	
Flow rate	0.6 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	70% B
5.0 – 6.0 min	70% B
6.0 – 7.0 min	10% B
7.0 – 11.0 min	10% B
Column temp	40°C
Autosampler	
Injection volume	2.0 µL
Injection flush-port	Active
Flush-port time/volume	15 sec
Flush-port solvent	75:25 HPLC grade MeOH: LC/MS H_2O

MASS SPECTROMETER

Ion mode	(+) MRM	Nebulizer gas	Nitrogen
Resolution	Unit	Nebulizer pressure	50 psi
Dwell	50	Drying gas	Nitrogen
Time Segment 1	To waste	Drying gas flow	12 L/min
Time Segment 2 (2 min)	To MS	Drying gas temp	350 °C
Time Segment 3 (6 min)	To waste	Capillary voltage	4.0 kV

Compound	MRM Transitions
Gabapentin-d_{10}	182.2 → 164.0, 147.1
Gabapentin	172.2 → 137.0, 95.0
Pregabalin-d_6	166.3 → 148.1, 103.1
Pregabalin	160.2 → 142.1, 97.1

LIST OF CHANGES

Revision Date	Description	Page Number
3/14/22	Method approved by Washington State Toxicologist. See DRA dated 1/19/2022. Method released for use in evidentiary testing on 3/14/22.	All