CONFIRMATION OF GABAPENTIN BY LIQUID CHROMATOGRAPHY- MASS SPECTROMETRY

35.1 POLICY

This test method may be used to confirm the presence of gabapentin (GABA) in biological samples. Quantitative results obtained through the use of this method will only be reported within the validated dynamic range. Reporting of results following the application of this method will be contingent upon a thorough review and acceptance of quality control data and the qualification of individual results under the criteria for acceptance.

Any adjustments or deviations from the procedures below must be approved by a member of TLD Management, and appropriately documented in the batch file.

35.2 PURPOSE

The purpose of this standard operating procedure (SOP) is to provide technical direction for the identification and quantitation of GABA present in biological specimens. This procedure will serve as the laboratory document describing sample preparation, instrumental analysis, data analysis, criteria for acceptance and reporting of the specified compound.

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35.3 PRINCIPLE

The targeted compound (GABA) and internal standard (GABA-d_{10}) are isolated from whole blood, serum, plasma, urine or other submitted biological samples by the use of protein precipitation and solid-phase extraction (SPE). Following SPE, the specimens, now termed extracts, are injected into a high performance liquid chromatograph (HPLC) where they are separated between a liquid mobile phase and a liquid stationary phase. Each compound exits the HPLC at a reproducible time which is termed its retention time.

The HPLC is coupled to a mass spectrometer (MS) detector equipped with an atmospheric pressure electrospray ionization source. As each ionized compound is drawn into the high vacuum region of the instrument, selected-ion monitoring is used to measure the mass-to-charge ratios of each compound and its related fragments. Multiple-point, internal standard calibration is used to generate a calibration curve. The concentration of any GABA identified in a sample is determined from its calibration curve.

35.4 SPECIMENS

35.4.1 The specimen volume is 0.5 mL.

35.4.2 Specimens include whole blood, serum, plasma, urine, and tissue homogenate.

NOTE: Matrix-matching of calibrators and controls is not required for quantitation of gabapentin in tissue homogenate samples, as determined

through evaluation of alternative matrix (liver homogenate) during method validation.

35.4.3 Dilutions of specimens may be analyzed at the Forensic Scientist's discretion; in addition, the specimen may be analyzed at standard volume, as dictated by screening results, to ensure that concentrations of the target compound present are within the dynamic range of the test method.

35.4.4 Analysis of larger specimen volumes must be approved and documented.

35.5 REAGENTS, MATERIALS AND EQUIPMENT

35.5.1 REAGENTS

35.5.1.1 Acetonitrile (ACN)

35.5.1.2 Ammonium hydroxide (NH_4OH), concentrated

35.5.1.3 Certified blank blood

35.5.1.4 Deionized water (DI H_2O)

35.5.1.5 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. Adjustments to final volume are permitted as long as the proportions of the elution solvent are maintained.

35.5.1.6 Formic acid (concentrated)

35.5.1.7 0.1% Formic acid

Add 1 mL of concentrated formic acid to 800 mL DI H_2O in a 1 L flask. Dilute to 1 L with DI H_2O and mix. Filter this solution prior to use on the HPLC. Store the acid in a glass bottle at room temperature for up to one year. Adjustments to final volume are permitted as long as the proportions are maintained.

35.5.1.8 Hydrochloric acid (HCl), concentrated

35.5.1.9 0.1M HCl

To 400 mL DI H_2O, add 4.2 mL concentrated HCl. Dilute to 500 mL with DI H_2O. Store the acid in a glass bottle at room temperature for up to 6 months. Adjustments to final volume are permitted as long as the proportions are maintained.

35.5.1.10 Methanol (MeOH)

35.5.2 MATERIALS

35.5.2.1 Autosampler vials (polypropylene, with integrated inserts) and caps

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35.5.2.2 Disposable 16 x 100mm tubes with closures

35.5.2.3 Disposable screw-cap tubes or centrifuge tubes with closures

35.5.2.4 Disposable pipette tips

35.5.2.5 Extraction column: United Chemical Technologies' Clean Screen SPE cartridge (CSDAU206 200mg/6mL), or equivalent

35.5.2.6 HPLC Column, Agilent Zorbax Eclipse Plus C18, 4.6 x 75 mm, dp = 3.5µM, or equivalent

35.5.2.7 Laboratory glassware (graduated cylinders, flasks)

35.5.2.8 Solvent filters (0.45 µm pore size; reduced cellulose, other)

35.5.2.9 Volumetric glassware (flasks)

35.5.3 EQUIPMENT

35.5.3.1 Agilent HPLC (1100/1200 series, or equivalent)

35.5.3.2 Agilent MS with API-ES source (SL model, or equivalent)

35.5.3.3 Calibrated, adjustable piston pipettes

35.5.3.4 Centrifuge

35.5.3.5 Evaporator (Caliper LS, formerly Zymark, TurboVap)

35.5.3.6 Solvent filtration apparatus

35.5.3.7 Vacuum manifold

35.5.3.8 Verified, adjustable repeater-pipette

35.5.3.9 Vortex mixer

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35.6 STANDARDS, CALIBRATORS AND CONTROLS

35.6.1 STANDARDS

35.6.1.1 Gabapentin reference material (referred to interchangeably in this method as stock standards) is used for the preparation of working standard (which in turn is used to produce calibrators and positive controls). The Gabapentin-d_{10} stock CRM is used directly in sample preparation.

35.6.1.2 Stock standards and stock internal standards are purchased from an approved reference material supplier and include the following:

a. Gabapentin: 1.0 mg/mL
b. Gabapentin-D_{10}: 0.1 mg/mL

35.6.1.3 Working standard (0.1 mg/mL)

a. Using a calibrated pipette, measure 2.5 mL GABA stock standard into a 25 mL class-A volumetric flask.
b. Add MeOH to the flask to the designated volume.
c. The final concentration of the working standard is 0.1 mg/mL. The working standard is stored in the freezer in an amber bottle and expires one year from the date of preparation (if a CRM expires prior to one year from date of preparation, the prepared solution expiration date becomes the first day of the month in which the CRM with the earliest expiration date expires). Volumes may be adjusted, provided that proportions remain constant.

35.6.2 CALIBRATORS

35.6.2.1 Calibrators are prepared in certified blank blood at the time of analysis using the working standard. The preparation of the calibrators is detailed in 35.7 SAMPLE PREPARATION. If necessary, calibrators may be prepared in alternate matrices provided that the matrix has been previously determined to not contain any of the compounds tested for by this procedure (see NOTE in 35.4.2). If the matrix has not been verified as negative, a matrix blank must be included in the batch.

35.6.3 CONTROLS

35.6.3.1 Negative Control

a. At least one negative whole blood control is tested with every batch. The negative control is prepared using certified blank blood.
b. When testing different sample types, wherever possible, include a negative control prepared from that matrix. For example, when analyzing whole blood and urine samples the batch shall include at least one negative whole blood control and at least one negative urine control (see NOTE in 35.4.2).

35.6.3.2 Positive Controls

a. At least two positive whole blood controls are tested with every batch. The positive controls are prepared using certified blank blood to which the designated volume of control working standard has been added.
b. Control stock standards are obtained from an approved reference material supplier.
c. The control stock standards must be either a different lot number or from a different supplier to those used in producing the working standard. If the same lot must be used, the working control standard must be prepared by someone other than the person that prepared the working standard.

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d. The control working standard (0.1 mg/mL) is prepared as described in 35.6.1.3.
e. The preparation of the positive whole blood controls is detailed in 35.7 SAMPLE PREPARATION. Alternatively, quality assurance personnel may provide in-house positive controls.
f. When testing different sample types, wherever possible, include at least one positive control prepared from that matrix (see NOTE in 35.4.2).

35.7 SAMPLE PREPARATION

35.7.1 Label a clean 16 x 100mm tube for each member of the test batch. (i.e. Calibrator, control, case sample).

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

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

a. Using a calibrated pipette, combine 0.1 mL of the working standard with 0.9 mL of ACN or MeOH in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after calibrator preparation.

35.7.4 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	Working Standard
Calibrator 1 – 1.0 mg/L	50	0.01 mg/mL
Calibrator 2 – 2.0 mg/L	100	0.01 mg/mL
Calibrator 3 - 5.0 mg/L	25	0.1 mg/mL
Calibrator 4 - 10 mg/L	50	0.1 mg/mL
Calibrator 5 - 15 mg/L	75	0.1 mg/mL
Calibrator 6 - 20 mg/L	100	0.1 mg/mL

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

Control Description	Volume (µL) Added	Control Working Standard
Control 1 – 3.0 mg/L	15	0.1 mg/mL
Control 2 - 16 mg/L	80	0.1 mg/mL

35.7.6 If in-house positive controls are being used, transfer 0.5 mL of each into their labeled tubes, using a calibrated pipette.

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

35.7.8 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.

35.7.9 Add 1 mL ACN to each tube.

35.7.10 Cap the tubes and briefly vortex mix. Centrifuge the tubes for 5 minutes at 2500-3000rpm.

35.7.11 Transfer the solvent layer to a new tube and add 2 mL 0.1M HCl. Vortex briefly.

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

35.7.13 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.

35.7.14 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.)

35.7.15 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.)

35.7.16 Dry the columns for 5 minutes under vacuum.

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

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

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

35.7.20 Reconstitute the extracts with the addition of 100 µL MeOH to each tube. Briefly vortex mix the tubes. If necessary, centrifuge the tubes for 2 minutes at 2000 rpm to collect the extracts at the bottom of the tubes.

35.7.21 Transfer the extracts to labeled polypropylene autosampler vials and cap.

35.8 INSTRUMENTAL PARAMETERS

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The instrumental parameters can be found in Appendix A. Prepare a batch worklist and set the data path in ChemStation/OpenLab to the date of the test. After entering all vial locations and sample descriptions in the sequence table, ensure that the method listing in the table is GABA for each line. As needed, the sequence may conclude with an injection that rinses the column (e.g., using method RINSE), or this may be done manually.

35.9 DATA ANALYSIS

35.9.1 Analysis of the batch data is conducted using ChemStation quantitative instrumental data analysis software.

35.9.2 Quantitative calculations are generated by internal standard, multi-point, linear regression with a 1/a (inverse of concentration) weighting factor. The calibration curve is updated using the calibrator results for the batch; no historical calibration curves are permitted.

35.9.3 Printed reports for each vial in the batch are generated for review along with the updated calibration curves and data analysis parameters (calibration report). Printed reports are not generated for injections used to equilibrate or rinse the HPLC column (e.g., using RINSE method).

35.9.4 Technical review of the batch is conducted according to the criteria listed below.

35.10 CRITERIA FOR BATCH ACCEPTANCE

If the analysis of the batch meets the criteria listed below, the results for the specimens are accepted.

35.10.1 CALIBRATORS AND CALIBRATION CURVES

35.10.1.1 Chromatographic peaks for GABA and internal standard shall appear symmetrical (i.e., no co-elution, split peaks, or shoulders).

35.10.1.2 Retention times for GABA and internal standard shall be within ±2% and ion ratios shall be within ±20% of those in calibrator 4. These are inclusive ranges.

35.10.1.3 Quantitative results for GABA in each calibrator shall be within ±20% of the target value with the exception of calibrator 1 which shall be within ±25% of the target. These are inclusive ranges. Result comparisons will use results truncated to one decimal place in units of mg/L.

35.10.1.4 The calibration curve for GABA shall have a correlation coefficient ≥0.99.

35.10.2 CONTROLS

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35.10.2.1 The negative control(s) shall not identify GABA above its limit of detection. Identification is based on a) acceptable retention time matching, b) distinct peaks present for all selected ions, and c) acceptable ion ratios.

35.10.2.2 Positive controls

a. Chromatographic peaks for GABA and internal standard shall appear symmetrical.
b. Retention times for GABA and internal standard shall be within ±2% and ion ratios shall be within ±20% of those in calibrator 4. These are inclusive ranges.
c. Quantitative results for GABA in each control shall be within ±20% of the target value. These are inclusive ranges. Result comparison will use results truncated to one decimal place in units of mg/L.
d. All positive controls in the batch must meet acceptability criteria for GABA in order to report quantitative results in a case specimen.

35.11 CRITERIA FOR CASE SAMPLE ACCEPTANCE

If the criteria for batch acceptance have been satisfied, the results of individual case samples are deemed suitable for reporting if the following criteria are met.

34.11.1 Any chromatographic peak for GABA and internal standard shall appear symmetrical.

34.11.2 The retention times for GABA and internal standard are within ±2% and the ion ratios are within ±20% of those in calibrator 4. These are inclusive ranges.

34.11.3 The quantitative result for GABA must be within the dynamic range of the test method. Results greater than the upper limit of quantitation may be reported qualitatively, provided that all other criteria for acceptance are met.

34.11.4 When dilutions of case samples are tested, the quantitative result(s) before multiplication shall be within the dynamic range of the test method.

35.12 REPORTING

35.12.1 Results are reported in units of milligrams per liter (mg/L).

35.12.2 Results are truncated to two significant figures for reporting.

a. Example 1: GABA is measured as 9.57 mg/L.

b. The result is truncated to 9.5 mg/L (two significant figures) and reported.

c. Example 2: GABA is measured as 14.38 mg/L.

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d. The result is truncated to 14.3 mg/L, but reported as 14 mg/L (two significant figures).

35.12.3 When multiple dilutions are analyzed, the smallest dilution within the dynamic range is reported.

35.13 METHOD PERFORMANCE

35.13.1 Limit of detection: 0.1 mg/L

35.13.2 Lower limit of quantification: 1.0 mg/L

35.13.3 Dynamic range: 1.0 – 20 mg/L

35.13.4 Upper limit of quantitation: 20 mg/L

35.13.5 Upper limit of linearity: 30 mg/L

35.14 TRACEABILITY

Traceability of the reference materials to SI units is provided through the certificate of analysis provided by the approved reference material supplier.

35.15 REFERENCES

35.15.1 Katie Harris and Dawn Sklerov, in house method development

35.15.2 A.F. Lehner, J. Stewart, A. Darfalla, 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)

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APPENDIX A
INSTRUMENTAL PARAMETERS

LIQUID CHROMATOGRAPH

Gradient Elution	
Flow rate	0.8 mL/min
Solvent A	0.1% Formic acid
Solvent B	ACN
Initial composition	90% A, 10% B
Hold time	5.0 min (10% 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

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	10	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

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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

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