SOP 406 - Gabapentin and Pregabalin Extraction using Protein Precipitation for Quantification by Liquid Chromatography/Electrospray Mass Spectrometry/ Mass Spectrometry (LC/MS/MS)

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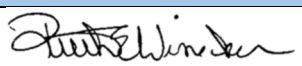
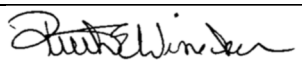
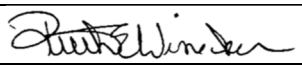
Approving Authority Name	Approving Authority Signature	Approval Date
Ruth E. Winecker, Ph.D.		04/17/2015
Ruth E. Winecker, Ph.D.		06/19/2016
Ruth E. Winecker, Ph.D.		12/07/2017

SOP 406 - Gabapentin and Pregabalin Extraction using Protein Precipitation for Quantification by Liquid Chromatography/Electrospray Mass Spectrometry/ Mass Spectrometry (LC/MS/MS)

1. **Principle of Assay**

    1.1. This method is designed to detect and confirm and quantitate Gabapentin and Pregabalin in biological specimens by Liquid Chromatography tandem Electrospray ionization Mass Spectrometry. The drugs are extracted from their biological matrix by protein precipitation with acetone and identified by the retention times of precursor ions and ion ratios of the product ions.

    1.2. Pregabalin and Gabapentin were released by the FDA for use in treating neuropathic pain and seizure disorders. The mechanism of action is not known at this time. The drugs are eliminated by systemic circulation and renal excretion as unchanged drug. High gabapentin/pregablin exposure can result in dizziness, ataxia, tachycardia, and hypotension. Patients with renal failure are at a greater risk of gabapentin/pregablin toxicity. Blood is the sample of choice for this assay though liver and urine may be used if no blood sample is available. Interpretation of postmortem levels must rely not only on drug concentration but also patient history, autopsy, and scene findings.

2. **Specimens**

    2.1. This procedure is applicable to blood, urine, serum, bile*, vitreous, properly prepared tissue specimens (typically 1:4 homogenates), and gastric contents*.

    2.2. A 0.1 mL (g) sample size in duplicate is generally employed, unless a dilution is required, so that the calibration curve encompasses the expected range of unknown specimens.

        2.2.1. *For non-typical matrices, an additional 0.1mL aliquot shall be taken (volume permitting), spiked with QC, and analyzed to help to identify any matrix effects. (See Non-Matched Matrix Protocol section of the QA/QC manual).

3. **Reagents and Materials**

    3.1. DI water, HPLC grade

    3.2. Methanol, HPLC grade

    3.3. Acetone, HPLC grade

    3.4. Acetonitrile, HPLC grade

    3.5. Internal Standard Mix

3.6. Gabapentin/Pregabalin Standard Mix

3.7. Gabapentin/Pregabalin QC Standard Mix

3.8. Drug Free Blood, Urine, Liver Homogenate

3.9. Water with 0.1% formic acid

3.10. Acetonitrile with 0.1% formic acid

4. **Standards, Controls, and Solutions**

4.1. **Pregabalin-d6 Stock Solution (100µg/mL)**

4.1.1. Into a 10mL volumetric flask, add the contents of 1 ampule (~1mL) of Pregabalin-d6 (Cerilliant - 1000µg/mL). Fill to the line with methanol, insert stopper and invert three times to mix. Transfer to properly labeled 16x100mm screw topped test tubes and cap. Store in laboratory refrigerator (R1-2601).

4.2. **Gabapentin-d10/Pregabalin-d6 Internal Standard (10µg/mL)**

4.2.1. Into a 10mL volumetric flask, add the following:

4.2.1.1. 1 ampule (~1mL) of Gabapentin-d10 (Cerilliant - 100µg/mL)

4.2.1.2. 1mL Pregabalin-d6 stock solution (100µg/mL)

4.2.2. Fill to the line with methanol, insert stopper and invert three times to mix. Transfer to properly labeled 16x100mm screw topped test tubes and cap. Store in laboratory refrigerator (R1-2601).

4.3. **Calibrators and Positive Controls –** these standards are to be prepared by the QA/QC Chemist or appointee. Inform the QA/QC Chemist if calibration/control standards need to be made.

4.4. **Water with 0.1% formic acid**

4.4.1. To a 4L bottle of HPLC grade water, add 4 mL of formic acid

4.4.2. Label bottle as “LC/MS” and “with 0.1% formic acid”.

4.5. **Acetonitrile with 0.1% formic acid**

4.5.1. To a 4L bottle of HPLC grade acetonitrile, add 4 mL of formic acid

4.5.2. Label bottle as "LC/MS" and "with 0.1% formic acid".

5. **Equipment and Special Supplies**

5.1. Test Tubes, , 16 x 125mm

5.2. LC autosampler vials, 12 x 32 mm

5.3. Polyspring inserts, 5 mm O.D.

5.4. Centrifuge 2000 x g

5.5. Vortex mixer

5.6. Nitrogen evaporator

6. **Instrumentation and Parameters**

6.1. Windows PC with Thermo LCQuan and Xcaliber software
6.1.1. Instrument method (TSQ01 & TSQ02): "Gabapentin"
6.1.2. Click here for instrument parameters.

6.2. Thermo Surveyor LC autosampler, or equivalent

6.3. Thermo Surveyor LC pump, or equivalent

6.4. Thermo TSQ triple quadrupole mass spectrometer

7. **Target Ions** (± 1 nominal mass)

7.1.	Gabapentin	(**172** 154 137)
7.2.	Gabapentin-d10	(**182** 164 147)
7.3.	Pregabalin-d6	(**166** 148 130)
7.4.	Pregabalin	(**160** 142 124)

7.5. Note: The precursor ion of each analyte is listed first and bolded, the first product ion- used for quantification-is second, followed by the second product ion-used for confirmation.

8. **Procedure**

8.1. Prepare a colored tape label for each standard, blank, control, and specimen to be placed on 16 x 125 mm test tubes.

8.1.1. **Note: follow the tube labeling and tape transfer procedure located in the Quality Assurance and Quality Control manual.**

8.2. Add the appropriate quantity (according to the Standard and Control Worksheet) of Pregabalin-d6 Internal Standard to all the tubes.

8.3. Add the appropriate quantity (according to the Standard and Control Worksheet) of the Gabapentin/Pregabalin Calibration Standard and the Gabapentin/Pregabalin QC Standard to the tubes labeled as standards and controls, respectively, labeling test tubes as you go. Only internal standard should be present in the test tube labeled "Blank".

8.4. Add 0.1mL of blank blood to all standards, control, and blank tubes. Include a urine blank/control and liver homogenate blank/control (as appropriate).

8.5. Add the appropriate amount of unknown specimen, labeling test tubes as you go. (See Specimens section).

8.6. Vortex all test tubes for 10 seconds.

8.7. Add 3.5mL acetone to each tube and vortex for 20 seconds.

8.8. Centrifuge at 2000 x g for 10 minutes.

8.9. Decant the top acetone layer into clean and labeled 13x100 test tubes, place in nitrogen evaporator, and evaporate at 55° C to dryness.

8.10. Remove dried specimens from nitrogen evaporator and reconstitute with 500 µL of methanol.

8.11. Vortex for 10 seconds and centrifuge at 2000 x g for 5 minutes.

8.12. Transfer ~100 µL of each extract to appropriately labeled autosampler vials fitted with 200 µL polyspring insert and place in the autosampler tray of the Thermo TSQ triple-quadrupole LC/MS/MS.

8.13. Build a sequence as directed in SOP 053.

9. Calculations

9.1. Quantitative Ion ratios

9.1.1. The method for processing the data using the Thermo LCQuan software is "Gabapentin" (SOP 055). It is used to calculate the internal standard response ratios, raw amounts, concentration and ion ratios.

9.1.2. These calculations are computed as follows:

9.1.2.1. Response Ratio:

9.1.2.1.1. Response Ratio = response of the analytes quantifying product ion compared to that of the internal standard's.

9.1.2.1.2. Response Ratio = QN_a / Qn_{istd}

9.1.2.1.2.1. QN_a = response of the quantitative ion of the analyte

9.1.2.1.2.2. QN_{istd} = response of the quantitative ion of the internal standard Amount

9.2. Dilution Factor

9.2.1. D = Total volume/Sample volume

9.3. Multiplier for homogenates, dilutions, and non-standard volumes

9.3.1. $M = (V_{curve} / V_{samp}) \times D$

9.3.1.1. M = Multiplier

9.3.1.2. D = dilution factor

9.3.1.3. V_{curve} = matrix volume of calibration curve

9.3.1.4. V_{samp} = matrix volume of specimen

9.4. Concentration

9.4.1. C = (A / V) * M

9.4.1.1. C = Concentration (ng/mL) of the analyte in the unknown case.

9.4.1.2. A = Amount of drug in sample

9.4.1.3. V = Volume of sample

9.4.1.4. M = Multiplier

9.5. Calibration

9.5.1. A linear regression resulting from the 5 standards is used to quantitate the analytes in the case. The area of the analyte divided by the area of the internal standard is used in the resulting formula of the calibration curve.

9.6. Max/Min

9.6.1. Percent Difference = $((R_h / R_l)-1) \times 100$

9.6.1.1. R_h = high result

9.6.1.2. R_l = low result

9.7. Average

9.7.1. Average = $(R_1 + R_2) / 2$

9.7.1.1. R_1 = first result

9.7.1.2. R_2 = second result

9.8. Qualifier Ion Ratios

9.8.1.1.1. Ratio 1 = QL_1/QN

9.8.2. QL_1 = response of the quantifying product ion

9.8.3. QN = response of the qualifying product ion

10. **Quality Control/Quality Assurance**

10.1. Acceptance criteria

10.1.1. Chromatogram

10.1.1.1. Peaks must be Gaussian shaped (symmetrical).

10.1.1.2. Peaks must not exhibit extreme fronting or tailing.

10.1.1.3. Peaks sharing parent/product ions must have baseline resolution.

10.1.1.4. Retention time must not deviate outside ± 3% of target, based upon the retention time of the calibrators and controls.

10.1.1.5. The internal standard (ISTD) in each case should be inspected for evidence of signal enhancement and suppression. The area of the quantifying ion should not be less than 50% or more than 200% of the average ISTD of the calibrators.

10.1.2. Mass spectrometry

10.1.2.1. The ion ratio of all samples must not be greater than ± 20% of the target ratio, as determined by a mid-level calibrator (CAL 3).

10.1.2.2. Coelution of quantifying and qualifying ions must not be greater than 0.025 minutes.

10.1.3. Calibrators

10.1.3.1. Analytical curves must have a coefficient of determination (R^2) of 0.992 or greater.

10.1.3.2. Each calibrator, when calculated against the calibration curve, must not deviate outside ± 20% of the target value (± 25% at the LOQ).

10.1.3.3. Refer to "Calibration curve point exclusion guidelines" section of the QA/QC Manual.

10.1.4. Controls

10.1.4.1. Controls must calculate within ± 20% of the target.

10.1.5. Blanks

10.1.5.1. Blanks should not contain any target analyte signal with an internal standard response ratio greater than 10% that of the lowest calibrator for the same analyte.

10.1.6. Any deviation from the above criteria must be approved by a senior chemist or toxicologist.

11. Validation of Method

11.1.

Parameter	Result
Bias	Gabapentin - Low: 0.74% High: 5.61% (Updated w/ Gaba-d10) Bias: -4.06% 6/15/201
	Pregabalin - Low: 1.10% High: 4.81%
Precision	Gabapentin - Low: 7.05% High: 5.48% (Updated w/ Gaba-d10) Bias: 7.19% 6/15/2015
	Pregabalin - Low: 4.45% High: 5.23%
Calibration model	Gabapentin - 1/x Linear Weighting
	Pregabalin - 1/x Linear Weighting
Carryover	Tested to 2X high calibrator with toxicologically insignificant amount of carryover. S/N threshold will be set to 800 (~ 30% of low calibrator). Any peak with S/N < 800 will be considered "none detected".
Interference Studies	No interfering signal observed
LOD (Calculate: 3.3xSD Y-intercept/Mean of Slope)	Administratively set at 0.2ug/mL (Calculated at 0.11ug/mL)
LOQ (Set to lowest calibrator with acceptable Accuracy/Precision).	1ug/mL
Processed Sample Stability - (re-analyze after 8 days)	Specimens determined to be stable for up to 1 week (Re-capped and stored refrigerated).

12. **Reporting**

12.1. The percent difference of duplicate analysis for an analyte must be less than or equal to 25% (see Max/Min in Calculations section).

12.2. Reporting of duplicate analysis should be done according to the table below:

Reporting Duplicates

- Dilution factors of 1 and 1

Dil / Scenario	1	1	REPORT
A	In curve	In curve	Average
B	In curve	AQL or BQL	"In" value
C	In curve	ND *	Repeat
D	AQL/BQL	AQL/BQL	Less than/ Greater than
E	BQL	ND	ND

* ND = None Detected, due to IRC, S/N threshold, r.t., or other

12.2.1.

12.2.1.1. In Curve = Measured concentration (pre-multiplier) falls within the calibration rang

12.2.1.2. AQL = Measured concentration (pre-multiplier) falls Above Quantitation Limit

12.2.1.3. BQL = Measured concentration (pre-multiplier) falls Below Quantitation Limit

12.2.1.4. ND = None Detected

12.3. Averaging reportable values

12.3.1. Results for duplicate analysis (both falling within calibration curve) shall be truncated prior to averaging.

12.3.2. Enter calculated average concentration for each specimen into toxlog.

12.4. Significant figures

12.4.1. Concentrations are truncated and reported with two significant figures in mg/L (maximum of three decimal places).

13. Preparation of Load

13.1. The load paperwork and data is to be arranged in the following order:

13.1.1. Assignment sheet

13.1.2. Comments or note to file if applicable

13.1.3. Load summary

13.1.4. Specimen worklist

13.1.5. Chain of custody (Specimen)

13.1.6. Aliquot chain of custody

13.1.7. Standard and control worksheet

13.1.8. Sequence summaries/calibration reports – paper clipped

13.1.9. Calibrator data - paper clipped

13.1.10. Blank matrix data - paper clipped

13.1.11. Control data - paper clipped

13.1.12. Specimen data – stapled

14. References

14.1. Mazzeo, J. R. Systematic and comprehensive strategy for reducing matrix effects in LC/MS/MS analyses. Journal of Chromatography B, 22-34.

14.2. Parke-Davis, comp. Neurontin®. 2998182. S.l.: S.n., 2007. Pfizer, 10 Aug. 2011. Web. June 2014. http://www.accessdata.fda.gov/drugsatfda_docs/label/2011/020235s050,020882s035,021129s033lbl.pdf

14.3. Klein-Schwartz, Wendy, J. Greene Shepherd, Susan Gorman, and Brad Dahl. "Characterization Of Gabapentin Overdose Using A Poison Center Case Series#." Clinical Toxicology 41.1 (2003): 11-15. Web.