SOP 203 - Acidic and Neutral Quantification by Solid Supported Liquid Extraction (SLE) and analysis by GC-FID.

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	9.1.2 – Updated RT acceptance range 9.1.3 – Updated QC acceptance range 9.1.8 – Updated Calibrator acceptance range	MSF – 05/11/2015
	Title – Corrected title read "quantification" instead of "screen" 1.1 – Corrected intro to read "quantitate" instead of "confirm"	MSF – 06/28/2016

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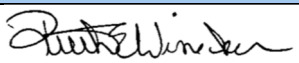
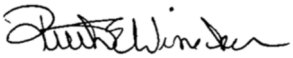
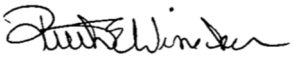
Ruth E. Winecker, Ph.D.		04/15/2015
Ruth E. Winecker, Ph.D.		11/02/2016
Ruth E. Winecker, Ph.D.		12/07/2017

SOP 203 - Acidic and Neutral Quantification by Solid Supported Liquid Extraction (SLE) and analysis by GC-FID.

1. Principle of Assay

1.1. This method is designed to detect and quantitate the presence of acidic and neutral drugs in blood, urine, tissue, and other specimens by GC-FID and GC/MS. The drugs are extracted from their biological matrix by solid supported liquid extraction and identified by their relative retention times and mass spectra.

2. Specimens

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

2.2. A duplicate specimen - aliquoted at dilution (lesser volume) - is generally employed so that the analyte concentration of the unknown specimen(s) falls within the range of the calibration curve (see Table 1). Specimens aliquoted at less than 1 ml/g will be supplemented to volume with blank blood/liver.

2.3.

Acid Neutral Quant

Specimen	Aliquot Volume
Blood	1.0mL / 0.2mL
Vitreous Humor	1.0mL / 0.2mL
Serum	1.0mL / 0.2mL
Plasma	1.0mL / 0.2mL
Urine/Bile*/Gastric*	0.5mL / 0.05mL
Liver Homogenate	0.5g / 0.05g
Other Tissue Homogenate*	1.0g / 0.2g

2.3.1. Table 1

2.4. *For non-typical matrices, an additional 1mL (g) 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. pH 5.00 potassium acid phthalate, sodium hydroxide buffer (Fisher Scientific)
3.2. Dichloromethane, HPLC grade
3.3. Acetonitrile, HPLC grade
3.4. Hexane, HPLC grade
3.5. n-Butyl Acetate, HPLC grade
3.6. Mephobarbital internal standard (100 µg/mL)
3.7. Standard 1, Standard 2, and QC Standard mixes.
3.8. Blood Bank Blood (drug-free)

3.9. Chem-Elut^{TM} 1003 Extraction Columns from Varian or equivalent

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

4.1. 100 µg/mL Mephobarbital Standard

4.1.1. In a 10 mL volumetric flask, pipette exactly 1 mL of a 1 mg/mL Mephobarbital stock solution with a micropipette. Dilute to the mark with methanol.

4.2. Acid/Neutral analyte calibration and control standards. Refer to SOP-010 for preparation instructions.

4.3. PEMA co-elutes with Mephobarbital. If the case contains Primidone (and PEMA) use another ISTD. Typically Pentobarbital is substituted as the ISTD in these cases.

5. **Equipment and Special Supplies**

5.1. 16x125 mm culture tubes
5.2. Vortex mixer
5.3. Centrifuge, capable of 2000 x g
5.4. Varian Bond Elut extraction columns
5.5. Positive Pressure Extraction Manifold
5.6. Nitrogen evaporation apparatus (Turbo-Vap)
5.7. 5mL conical tubes
5.8. 2mL Auto sampler vials
5.9. 0.2mL polyspring inserts
5.10. Crimp caps for 2mL Auto sampler vials

6. **Instrumentation and Parameters**

6.1. Agilent
6.1.1. Windows PC with Agilent Chemstation software
6.1.2. Agilent 7683 autosampler; or equivalent
6.1.3. Agilent 6890 Gas Chromatograph with 5973 Mass spectrometer (GC/MS); or equivalent
6.1.4. Agilent 6890 Gas Chromatograph with Nitrogen Phosphorous Detector (GC/NPD); or equivalent

6.2. Thermo
6.2.1. Windows PC with Thermo Xcalibur and Tox Lab Forms software
6.2.2. Thermo Trace GC Ultra Gas Chromatograph with FID; or equivalent
6.2.3. Thermo DSQ/DSQ II Mass Spectrometer; or equivalent
6.2.4. Thermo Triplus autosampler; or equivalent

7. Procedure

7.1. FID Procedure

7.1.1. Prepare a colored tape label for each standard, blank, control, and specimen to be placed on 16 x 100 culture tubes.

7.1.2. Prepare standards and controls as outlined on the appropriate Standard & Control Worksheet.

7.1.3. Pipette 1 mL of negative (drug free) control blood into all standard, blank, and QC controls.

7.1.4. Pipette a quantity of specimen with a micropipetter into 16 X 125mm test tubes labeled with the case number, labeling test tubes as you go.

7.1.4.1. The amount of specimen will be determined by the specimen type as described in the Specimens section of this procedure.

7.1.5. Add 1 mL of the pH 5.00 buffer and vortex for 5 seconds.

7.1.6. Install the "Extraction Plate" atop the SPE waste tank.

7.1.7. Place the appropriate number of Chem Elut^{TM} columns into the "Extraction Plate".

7.1.8. Place the SPE waste tank onto a Positive Pressure Extraction Manifold

7.1.9. One sample at a time, transfer the label tape from a specimen test tube to a clean 16X125mm test tube.

7.1.10. Pour the sample into one of the Chem Elut^{TM} columns (7.1.7).

7.1.11. Place the labeled (clean) test tube into an SPE rack in the corresponding location to the Chem Elut^{TM} column used.

7.1.12. Repeat 7.1.9 - 7.1.11 for all specimens.

7.1.13. Wait 2 minutes after the samples reach the frit of the column. Apply 2-5psi (low) positive pressure as needed.

7.1.14. Remove the "Extraction Plate" containing the Chem Elut^{TM} columns from the waste tank and place onto the SPE rack so that the nozzle of the Chem Elut^{TM} columns rests in its corresponding 16X125mm test tube.

7.1.15. Add 5 mL of dichloromethane to each column and wait 2 minutes after the solvent reaches the frit of each column. Apply 2-5psi (low) positive pressure as needed.

7.1.16. Repeat 2X until a total 15mL of dichloromethane has been added to each column.

7.1.17. Apply 2-5psi (low) positive pressure as needed to extract as much elution solvent (dichloromethane) as possible.

7.1.18. Evaporate each sample under a stream of nitrogen at 40°C to dryness. (Approximately 18-20 min).

7.1.19. Reconstitute each sample with 125 µL of acetonitrile using a micropipetter,

7.1.20. Vortex for 10 seconds

7.1.21. Transfer to 5 mL conical tubes.

7.1.22. Add 500 µL of hexane and vortex for 10 seconds.

7.1.23. Centrifuge at 2000 x g for 5 minutes.

7.1.24. Aspirate the top hexane layer (1)

7.1.25. Note: It is not necessary to remove all of the hexane at this step.

7.1.26. Add 500 µL of hexane and vortex for 10 seconds.

7.1.27. Centrifuge at 2000 x g for 5 minutes.

7.1.28. Aspirate the top hexane layer (2)

7.1.29. Note: It is not necessary to remove all of the hexane at this step.

7.1.30. Add 500 µL of hexane and vortex for 10 seconds.

7.1.31. Centrifuge at 2000 x g for 5 minutes.

7.1.32. Carefully, aspirate all the top hexane layer without removing any of the acetonitrile layer.(3)

7.1.33. Transfer ~50 µL of the acetonitrile to the appropriately labeled autosampler vial with a micropipetter.

7.1.34. Build a sequence on the instrument to be utilized. Print the sequence and review it for errors.

7.1.35. Place the autosampler vials into the appropriate positions in the autosampler trays of the GC/FID or GC/MS and have another analyst verify the position of the vials in the auto sampler trays.

7.1.36. Initiate the sequence and begin injecting.

7.1.37. After completion of the sequences on the GC/FID or GC/MS, transfer the samples to the other instrument (e.g. samples analyzed on GC/FID are transferred to GC/MS or vice versa) and repeat steps 7.1.34- 7.1.36.

7.2. Alternate procedure for injection of specimens on GC-NPD:

7.2.1. Follow steps 7.1.1 - 7.1.32

7.2.2. Evaporate each sample under a stream of nitrogen at 60°C to dryness.

7.2.3. Reconstitute each sample with 100 µL of n-Butyl Acetate and vortex for 10 seconds.

7.2.4. Transfer ~50 µL of the n-Butyl Acetate to the appropriately labeled autosampler vial with a micropipetter.

7.2.5. Proceed with steps 7.1.34 - 7.1.37 above.

8. **Calculations**

8.1.1. Data analysis software is used to calculate the internal standard response ratios, raw amounts, and concentration. It is also used to calculate the qualifier ion ratios.

8.1.2. These calculations are computed as follows:

8.1.2.1. Response Ratio:

8.1.2.1.1. Response Ratio = response of the analytes quantifying product ion compared to that of the internal standards.

8.1.2.1.2. Response Ratio = QN_a / Qn_{istd}

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

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

8.2. Calibration

8.2.1. A linear regression resulting from the 6 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.

8.3. Dilution Factor

8.3.1. D = Total volume/Sample volume

8.4. Multiplier for homogenates/dilutions and non-standard volumes

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

8.4.1.1. M = Multiplier

8.4.1.2. D = dilution factor

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

8.4.1.4. V_{samp} = matrix volume of specimen

8.5. Concentration

8.5.1. C = (A / V) * M

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

8.5.1.2. A = Amount of drug in sample

8.5.1.3. V = Volume of sample

8.5.1.4. M = Multiplier

8.6. Max/Min

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

8.6.1.1. R_h = high result

8.6.1.2. R_l = low result

8.7. Average

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

8.7.1.1. R_1 = first result

8.7.1.2. R_2 = second result

9. **Quality Control**

9.1. For an analysis to be acceptable the following criteria must be met:

9.1.1. Chromatography must be acceptable with a symmetrical (Gaussian) shape. Each analyte of interest must have near baseline resolution from any other peaks in the chromatogram (FID).

9.1.2. The retention time of each analyte should be within ± 2% of the expected retention time based on the calibrators and the relative retention time to the internal standard.

9.1.3. The quality control samples shall have an analytical value as stated on the standard and control worksheet, and shall not deviate ± 20% from the expected value.

9.1.4. 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.

9.1.5. The internal standard areas of the samples should be within 50-200% of the internal standard areas of the calibrators, control, and blank.

9.1.6. Results must fall within the linear range of the assay. Results above or below the linear range (higher than the highest calibrator used or lower than the lowest calibrator used, respectively), can only be reported as “less than” the lowest calibrator or “greater than” the highest calibrator.

9.1.7. The calibration curve shall have a R2 > 0.992

9.1.8. The calibrators shall not deviate from target concentration ± 20% (± 25% at LOQ)

9.1.9. If conditions in 9.1.1 - 10.1.8 are not met, consult with a supervisor or senior chemist for solutions.

10. **Validation of Method**

10.1. Pending

11. **Reporting**

11.1. **Refer to SOP-054 (Agilent) and SOP-055 (Thermo) for data processing procedures.**

11.2. For an analyte to be reported as present (qualitatively or quantitatively) in a case specimen, chromatographic and mass spectral guidelines must be met (see QA/QC Manual).

11.3. Qualitative results: Reported as "Present".

11.3.1. Analyte must be identified in both MS and GC-FID analysis.
11.3.2. Analyte is not included or did not pass acceptance criteria in QC samples.

11.4. Quantitative results:

11.4.1. Analyte must be identified in both MS and GC-FID.

11.4.2. Analyte is included and passed acceptance criteria in QC samples.

11.4.3. Results must fall within the linear range of the assay. Results below or above the linear range can only be reported as "less than" the lowest used calibrator or "greater than" the highest used calibrator.

11.4.4. Results are reported with two significant figures. All results will be truncated (not rounded) to the nearest significant figure.

12. **Reinjection Protocol**

12.1. A sample may be reinjected due to autosampler failure, apparent low recovery, to check for carry-over or to meet ion ratio and/or retention time criteria. Reinjected sample(s) must be followed by reinjection of either the duplicate

case sample(s) or matrix-matched calibrator or control. All reinjected samples must meet QA/QC criteria.

12.2. See the QA/QC Manual (Section 2.15) for laboratory guidelines.

13. **Load Assignment Packet Preparation**

13.1. After completing all data generation and reviewing for corrections, the analyst will assimilate the data in the following order:

13.1.1. Load assignment sheets, followed by any additional notes to file pertaining to load.
13.1.2. Load specimen sheet.
13.1.3. Chain of Custody (electronic)
13.1.4. Aliquot Chain of Custody
13.1.5. Standard and Control Worksheet.
13.1.6. GC/FID Method Calibration.
13.1.7. GC/FID and GC/MS Running Sequences.
13.1.8. Data Analysis Printouts with current area and/or ion ratios for standards 1 and 2.
13.1.9. GC/FID and GC/MS data for Standard 1, Standard 2, Standard 3, Blank, QC1 (low) and, QC2 (high) and QC0.5 (ultralow)
13.1.10. GC/FID and GS/MS data for assigned specimens.
13.1.11. The Load Checklist should be initialed and dated to acknowledge completion of load. The finished data package will be placed in the data review box in room 2401.

14. **References**

14.1. Levine, Barry. "Postmortem Forensic Toxicology." *Principles of Forensic Toxicology*. 2nd ed. Washington, DC: AACC, 2006. 67-79. Print.

14.2. Bishop-Freeman, Sandra C.; Kornegay, Nina C.; Winecker, Ruth E. *Postmortem Levetiracetam* (*Keppra®) Data from North Carolina.* J Anal. Toxicol. July 36:422–428 (2012).

14.3. JL Poklis, JD Ropero-Miller, D Garside and RE Winecker: *Case Report: Metaxalone (Skelaxin ®) Related Death.* J Anal Toxicol. Oct; 28:537-541 (2004).

14.4. Anderson, William and Dwain Fuller. A Simplified Procedure for the Isolation, Characterization, and Identification of Weak Acid and Neutral Drugs from Whole Blood. *Journal of Analytical Toxicology*, 198-204 (1987).

14.5. Anderson, William and H.S. Nichols. The Characterization of Weak Acid and Neutral Drugs by Capillary Column Gas Chromatography.