SOP 103 - Acidic and Neutral Screen by Solid Supported Liquid Extraction (SLE) and analysis by GC/MS and FID.

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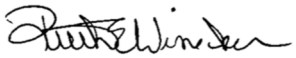
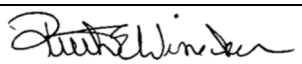
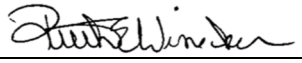
North Carolina Office of the Chief Medical Examiner Toxicology Laboratory	**Revision:**	**Revision Date/Initials:**
	10. – Inserted validation table	MSF – 04/04/2016
	7.1.32 – Updated sequence parameters 9.1.3 – Updated QC criteria for low QC.	MSF – 09/28/2016
	11.3-11.9 – Updated reporting instructions	MSF – 10/10/2016
	7.1.1 – Updated list of positive controls (Ultra Low QC) 7.1.32.1 – Updated sequence order 9.1.4 – Added acceptance criteria for Ultra-low QC 9.1.5 – Updated negative control acceptance criteria 11 – Updated reporting section	MSF – 03/06/17
	4 – Added instruction for preparing drug standards 6 – Updated instrumentation/methods	MSF – 05/24/2017 MSF - 6/8/2017
	7.1.6 – Added Centrifugation Step	MSF – 11/7/2017

Approving Authority Name	Approving Authority Signature	Approval Date
Ruth E. Winecker, Ph.D.		04/08/2015
Ruth E. Winecker, Ph.D.		06/19/2016
Ruth E. Winecker, Ph.D.		12/06/2017

SOP 103 - Acidic and Neutral Screen by Solid Supported Liquid Extraction (SLE) and analysis by GC/MS and FID.

1. **Principle of Assay**

    1.1. This method is designed to detect and confirm 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. Blood, urine, vitreous, bile, gastric contents, or tissue homogenate - volume varies depending upon drug concentration and specimen availability, typically 1 mL of blood, urine, vitreous, bile, or gastric contents, or 1 g of tissue homogenate (1:4 dilution).
    2.2. Specimens aliquoted at less than 1 ml/g will be supplemented to volume with blank blood/liver.

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™ 1003 Extraction Columns from Varian.

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

    4.1. Mephobarbital Stock Solution (1mg/mL) - as the Mephobarbital stock material is a controlled substance, it will be prepared by the QA/QC Chemist or appointee. Inform the QA/QC Chemist if the Mephobarbital stock solution needs to be made.

4.2. Mephobarbital Internal Standard (100 µg/mL)

4.2.1. 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, insert stopper and invert three times to mix. See SOP-010.

4.2.2. Alternatively, 1ml from an ampule of 1mg/mL Mephobarbitol may be used to prepare the Internal Standard solution.

4.3. Standard 1, Standard 2, and QC Standard mixes - 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.

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 –

6.1.3.1. Use the 13ANSCRN.M method.

6.1.3.2. Click GC/MS13 for GC/MS parameters.

6.1.4. Agilent 6890 Gas Chromatograph with Flame Ionization Detector (GC/FID); or equivalent.

6.1.4.1. Use the ANFID.M method.

6.1.4.2. Click\\mefs01\share\toxicology\QAQC\SOP\Instrument Methods\Agilent OB npd2.pdf NPD03 for GC/FID parameters.

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; e.g. Standard 1, Standard 2, Blank, QC Low, and QC Ultra-low.

7.1.2. Prepare standards and controls as outlined on the Acid-Neutral 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 micropipette 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. Centrifuge samples for 5 minutes at 2000 x g.

7.1.7. Install the “Extraction Plate” atop the SPE waste tank.

7.1.8. Place the appropriate number of Chem Elut™ columns into the “Extraction Plate”.

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

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

7.1.11. Pour the sample into one of the Chem Elut™ columns (7.1.8).

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

7.1.13. Repeat 7.1.10 - 7.1.12 for all specimens.

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

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

7.1.16. 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.

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

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

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

7.1.20. Reconstitute each sample with 125 µL of acetonitrile using a micropipetter or a repeating pipettor set to 2.5 with green repeater tips (1=50µL).

7.1.21. Vortex for 10 seconds

7.1.22. Transfer to 5 mL conical tubes using disposable Pasteur pipettes.

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

7.1.24. Centrifuge at 2000 x g for 5 minutes.

7.1.25. Aspirate the top hexane layer (1)

7.1.25.1. 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)

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

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

7.1.30. Centrifuge at 2000 x g for 5 minutes.

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

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

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

7.1.33.1. Place urine, fatty, tissue, and decomposed specimens at end of sequence (following positive QCs) to prevent fouling the GC inlet/column.

7.1.33.2. The order of specimens in the analytical sequence shall reflect the following:

7.1.33.2.1. Test mix (Std. 1 neat)
7.1.33.2.2. QC Ultra-Low
7.1.33.2.3. Standard 1
7.1.33.2.4. Standard 2
7.1.33.2.5. Case Specimens (clean)
7.1.33.2.6. QC Low
7.1.33.2.7. QC Ultra-Low
7.1.33.2.8. Case Specimens (fatty - 7.1.33.1)

7.1.33.3. **Note**: the QC Ultra-Low shall be injected at the beginning and at the end of the analytical run (see 9.1.4)

7.1.34. 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.

7.1.35. Initiate the sequence and begin injecting.

7.1.36. 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 7.1.33- 7.1.35.

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

7.2.1. Follow steps 7.1.1 - 7.1.31

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 micropipette.

7.2.5. Proceed with steps 7.1.33 - 7.1.36 above.

8. **Calculations**

8.1. Internal Standard Response Ratio:

8.1.1. Response Ratio = Aa / Aistd

8.1.2. Response Ratio = response of the analyte compared to that of the internal standard

8.1.3. Aa = area of the analyte

8.1.4. Aistd = area of the internal standard

8.1.5. Relative Retention Time:

8.1.5.1. RRT = RTa / RTistd

8.1.5.1.1. RRT = relative retention time

8.1.5.1.2. RTa = retention time of the analyte

8.1.5.1.3. RTistd = retention time of the internal standard

8.2. Raw Amount

8.2.1. Analyte = (Aa/Aistd) / (SAa/SAistd)

8.2.1.1. Analyte = µg of analyte in unknown case

8.2.1.2. Aa = area of drug in unknown case

8.2.1.3. Aistd = area of internal standard in unknown case

8.2.1.4. SAa = area of drug in standard

8.2.1.5. SAistd = area of internal standard in standard

8.3. Adjusted Amount

8.3.1. Analyte = {(Aa/Aistd) / (SAa/SAistd)} * D

8.3.1.1.1. Analyte = µg of analyte in unknown case (adjusting for dilution)

8.3.1.1.2. Aa = area of drug in unknown case

8.3.1.1.3. Aistd = area of internal standard in unknown case

8.3.1.1.4. SAa = area of drug in standard

8.3.1.1.5. SAistd = area of internal standard in standard

8.3.1.1.6. D = dilution factor

8.4. Concentration

8.4.1. Analyte = {[(Aa/Aistd) / (SAa/SAistd)} / V] * D

8.4.1.1.1. Analyte = µg/mL of analyte in unknown case

8.4.1.1.2. Aa = area of drug in unknown case

8.4.1.1.3. Aistd = area of internal standard in unknown case

8.4.1.1.4. SAa = area of drug in standard

8.4.1.1.5. SAistd = area of internal standard in standard

8.4.1.1.6. V = volume of sample

8.4.1.1.7. D = dilution factor

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 ±5% 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 (QC Low) shall have an analytical value as stated on the standard and control worksheet, and shall not deviate ±30% from the expected value.

9.1.4. The QC Ultra-Low (both pre and post-run) must meet acceptance criteria for chromatography and retention time in order to report an associated analyte in a means other than qualitatively.

9.1.5. Blanks should not contain any target analyte signal above the limit of detection (Area response ratio must be <10% that of low QC).

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

9.1.7. If conditions in 9.1.1 - 9.1.6 are not met, consult with a supervisor or senior chemist for solutions.

10. **Validation of Method**

Parameter	**Result**
Bias at decision point (Low Control)	All analytes evaluated displayed Bias of <20%.
Precision at decision point	All analytes evaluated displayed %CV of <20% with the exception of phenytoin (23.47% @ 4 mg/L).
Calibration model	One point calibration with a positive control at decision point (low control) and another at Upper Limit of Quantitation.

Carryover	No analytes were detected following injection of calibration standards. In any case specimen in which an analyte peak is greater than approximately 3X that of IS, the following specimen will be evaluated for the presence of possible carryover.
Interference Studies	Matrix interference will be determine on a per specimen basis - Data sources are full scan MS and FID therefore, detection of unknown analytes is desirable for the purpose of this assay. IS (Mephobarbital) recovery that is outside 50-200% of calibrators and control values will be investigated.
LOQ (Set to low QC calculated values) - See Standard and control worksheet.	Analytes that fall below LOQ to be reported as "less than (LOQ)" those that calculate above LOQ will be Confirmed and Quantitated at Toxicologist's discretion. - (Exceptions noted on Std & Ctrl worksheet).
LOD	Not Determined.

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 Chromatographic and Mass Spectral Quality Control section of QA/QC Manual).

11.3. Only analytes that are present in both GC-FID and GC/MS chromatograms will be reported. See the QA manual for Guidelines for Mass Spectrometer Peak Identification. (See SOP 054).

11.3.1. If an acid/neutral peak(s) is only detected on one data set, MS or FID, do not report but label as outlined below:

11.3.1.1. If an analyte is only detected on the FID – label with "NI" (not identified).

11.3.1.2. If an analyte is only detected on the GCMS – label as "c/w" (consistent with) – **unless the analyte is phenobarbital and/or phenytoin** and was detected in the previously injected specimen – in which case label with analyte name followed by "CO" (carryover).

11.3.2. If an acid/neutral peak(s) is detected on both MS and FID, but its integrated area count is ~ ¼ that of the low QC (or less) with poor peak shape and/or a low MS quality match, the chromatographic peaks on both MS and FID data sets shall be labeled with a "c/w" and **not** reported.

11.3.3. If an acid/neutral peak(s) is detected on both MS and FID, with an integrated area count of > ~ ¼ that of the low QC and meets reporting criteria as outlined in section 11.3, the chromatographic peaks on both MS and FID data sets shall remain labeled and the analyte(s) shall be reported (see 11.6 - 11.7).

11.4. All reportable compounds detected in urine shall be reported as "Present".

11.5. If caffeine, lidocaine, levamisole, diazepam or other drugs typically reported on the organic Base Screen (SOP 102) are detected, label as appropriate and do not report.

11.6. For drugs that are represented in the Standards/QCs:

11.6.1. If the calculated drug concentration is < the low QC, report as follows:

11.6.1.1. If the QC Ultra-Low meets acceptance criteria (11.2), report as "Less Than 'Low QC Level'"

11.6.1.2. If the QC Ultra-Low does not meet acceptance criteria (11.2), report as "Present"

11.6.2. If the calculated drug concentration is greater than the associated low QC concentration, do not report – the reviewer will decide how to evaluate the result.

11.6.3. If the associated low QC fails acceptance criteria for a detected analyte, report as "Present" (11.6.1) or do not report (11.6.2) using reporting criteria described above with the notation "QC Fail" adjacent to the analyte's calculated concentration on the report.

11.7. For detected acidic or neutral drugs that are not represented in the Standards/QCs; report as "Present".

11.8. In addition to reporting the drug analytes, the following shall be reported with each case.

11.8.1. If no organic acids or neutrals are detected – report "Organic Acids/Neutrals - None Detected"

11.8.2. If all detected organic acids and/or neutrals are being reported; report "Other Organic Acids/Neutrals – None Detected".)

11.8.3.

11.8.4. If detected drugs are not being reported (11.6.2); report "Other Organic Acids/Neutrals – Present".

12. **Reinjection**

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 the QA/QC criteria.

12.2. See the QA/QC Manual 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. 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. 14.5 Anderson, William and H.S. Nichols. The Characterization of Weak Acid and Neutral Drugs by Capillary Column Gas Chromatography.