102 - Organic Base Screen by Liquid/Liquid Extraction and analysis by GC/MS and NPD.

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SOP Name:	SOP #:
Organic Base Screen by Liquid/Liquid Extraction and analysis by GC/MS and NPD.	**102**

	Revision:	Revision Date/Initials:
North Carolina Office of the Chief Medical Examiner Toxicology Laboratory	9.1.5 – IS acceptance range updated	MSF – 04/14/2015
	10.1 – Validation Table added	MSF – 04/14/2015
	2.1 - 2.1.1 – Specimen list updated	MSF – 01/11/2016
	3.9, 7.3, 12.1.9 – Removed reference to “Standard 3”.	MSF – 09/27/2016
	7.28.1 – Added QC Ultra Low sequence info.	MSF – 09/28/2016
	9.1.3 – Added acceptance criteria for QC Ultra Low	
	4 – Added instructions for preparing drug standards	MSF – 05/24/2017
	6.131 & 6.141 – Updated Method hyperlinks	MSF – 05/26/2017
	6.2 – Deleted Thermo info	
	2.1.1 – Updated Specimen table	MSF – 06/22/2017

Approving Authority Name	Approving Authority Signature	Approval Date
Ruth E. Winecker, Ph.D.	Ruth E Winecker	04/08/2015
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1. **Principle of Assay**

   1.1. This method is designed to detect the presence of basic drugs and poisons in blood, urine, and tissue specimens by GC-NPD and GC/MS. The drugs are extracted from their biological matrix by liquid-liquid extraction and identified by retention order/RRT/RI and mass spectra.

2. **Specimens**

   2.1. Typically, blood specimens are assigned for organic base screening, however, when not available for testing, other specimens such as urine, vitreous, tissue, and other cadaveric fluids may be extracted. Specimen volumes used for testing vary and depend on specimen availability; the standard volume used when screening of organic bases is as follows:

Organic Base Screen

Specimen	Aliquot Volume
Blood	2.0 mL
Vitreous Humor	1.0 mL
Serum	2.0 mL
Plasma	2.0 mL
Cadaveric Fluid	2.0 mL
Urine/Bile	1.0 mL
Tissue homogenate/Gastric	1.0 g
Infant Tissue homogenate *(denoted by "##" on assignment sheet)*	2.0g

2.1.1. Table1

3. **Reagents and Materials**

   3.1. n-Butyl Chloride, HPLC grade
   3.2. Ethyl ether, HPLC grade
   3.3. Concentrated Ammonium Hydroxide
   3.4. Water, HPLC grade
   3.5. 2N Sulfuric Acid
   3.6. Hexane, HPLC grade
   3.7. n-Butyl Acetate, HPLC grade
   3.8. Alphaprodine Internal Standard
   3.9. Standard 1, Standard 2, and QC Standard Mix
   3.10. Drug Free Blood (matrix)

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

4.1. **Alphaprodine stock solution (1mg/mL)** – as the Alphaprodine stock material is a controlled substance, it will be prepared by the QA/QC Chemist or appointee.

4.2. **Alphaprodine working solution (Internal Standard - 20 µg/mL)**

  4.2.1. With a 1000µL micro pipette, transfer 1mL of Alphaprodine stock solution (1mg/mL) into a 50mL volumetric flask. Fill to the line with methanol, insert stopper and invert three times to mix. Transfer to properly labeled 16x125mm screw topped test tubes and cap. Store in laboratory refrigerator (R1-2601). See SOP-010

4.3. **Calibrators and Positive Control 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.

4.4. **3:1 n-Butyl Chloride:Ethyl Ether**

  4.4.1. To a clean and dry 4 liter solvent bottle place 3 liters n-butyl chloride. Add 1 liter diethyl ether. Mix well.

  4.4.2. Alternatively, in a 1 liter graduated cylinder, place 750 mL of n-butyl chloride. Add 250 mL of ethyl ether. Mix well.

4.5. **2 N Sulfuric Acid**

  4.5.1. From a new 4 liter bottle of deionized water, remove 232 mL. Slowly add 232 mL of concentrated sulfuric acid. Replace cap and mix gently.

  4.5.2. Alternatively, in a 500 mL volumetric flask, place approximately 300 mL of deionized water. Slowly add 29 mL of concentrated sulfuric acid and gently vortex. Dilute to the mark with deionized water. Mix well.

5. **Equipment and Special Supplies**

5.1. Vortex Mixer
5.2. Centrifuge
5.3. Micro Pipette, air displacement 10-100 µL, calibrated
5.4. Micro Pipette, positive displacement 100-1000 µL, calibrated
5.5. Micro Pipette, air displacement 100-1000µL, calibrated
5.6. Micro Pipette, positive displacement 10-100 µL, calibrated
5.7. Vacuum assisted aspirator
5.8. Pasteur pipettes
5.9. 16X125 screw top test tubes
5.10. 5mL conical test tubes
5.11. Linerless polypropylene caps (15-415 & 13-415 thread size)
5.12. 2mL GC Autosampler vials
5.13. 0.1mL polyspring inserts

5.14. Crimp caps for 2mL GC Autosampler 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 "#SplitOB.M" method (# = Instrument Number)
            - 6.1.3.2. Click GC/MS13, GC/MS14, or GC/MS15 for GC/MS parameters
        - 6.1.4. Agilent 6890 Gas Chromatograph with Nitrogen Phosphorous Detector (GC/NPD); or equivalent
            - 6.1.4.1. Use the "HOBSCRN.M" method
            - 6.1.4.2. Click NPD02, NPD03, NPD04 for GC/NPD parameters

7. **Procedure**

7.1. Prepare a colored tape label for each standard, blank, control, and specimen to be placed on 16x125 mm test tubes.

7.2. To the appropriate number of 16X125 screw topped test tubes, add 100 µL of 20 µg/mL Alphaprodine internal standard solution. See Standard and Control Worksheet.

7.3. Add 100 µL of Standard 1 mix to the blood bank control labeled "Standard 1"; Add 100 µL of Standard 2 mix to the blood bank control labeled "Standard 2"; Add 50 µL of QC Low mix to the blood bank control labeled "QC Low"; Add 25 µL of QC Low mix to the blood bank control labeled "QC Ultra Low", labeling test tubes as you go.

7.4. Pipette 2 mL of blank blood into all standard, blank, and QC samples.

7.5. Pipette the appropriate volume of specimen as determine in Table1 (unless otherwise directed upon assignment) into 16x125mm glass screw top test tubes labeling test tubes as you go.

7.6. Add 0.5 mL Concentrated Ammonium Hydroxide to each sample and vortex for 10 seconds.

7.7. Add 7 mL 3:1 n-butyl chloride/ethyl ether mixture to each tube.

7.8. Cap each tube and shake vigorously for 3 minutes, or place on rotator for 15 minutes.

7.9. Centrifuge for 10 minutes at 2000 x g.

    - 7.9.1. Note: if an emulsion or gel forms at this stage (indicated by a tri-layer or lack of recovery of most of the 3:1 mixture), add 1-2 mL of ether to the tube, shake or rotate the required time and then re-centrifuge.

7.10. Transfer the top organic layer to a new 16x125 mm glass culture tube.

7.11. Add 2.5 mL of 2N sulfuric acid.

7.12. Cap each tube and shake vigorously for 3 minutes, or place on rotator for 15 minutes.

7.13. Centrifuge for 10 minutes at 2000 x g.

7.14. Aspirate the top n-butyl chloride/ethyl ether layer to waste.

7.15. Add 2 mL of hexane to the remaining aqueous layer.

7.16. Cap each tube and shake vigorously for 3 minutes, or place on rotator for 15 minutes or vortex without capping for 20 seconds.

7.17. Centrifuge for 5 minutes at 2000 x g.

7.18. Aspirate the top hexane layer to waste.

7.19. Add 1 mL Concentrated Ammonium Hydroxide to the remaining aqueous layer.

7.20. Vortex for 10 seconds.

7.21. Transfer each sample to a 5 mL screw cap conical tube.

7.22. Add 100µL n-butyl acetate to each sample with a repeat pipettor.

7.23. Vortex for 20 seconds.

7.24. Centrifuge for 5 minutes at 2000 x g.

7.25. With a Pasteur pipette, withdraw all but about 100 µL of the bottom aqueous layer to waste.

7.26. Centrifuge at 2000 x g for 5 minutes.

7.27. Transfer ~50 µL of the top organic layer to an appropriately labeled autosampler vial with a micropipette.

7.27.1. NOTE – Transfer of ANY of the bottom aqueous layer into the autosampler vial will result in irreparable damage to the GC column.

7.28. Build a sequence on the instrument to be utilized (SOP 050). Print the sequence and review it for mistakes.

7.28.1. The order of specimens in the analytical sequence shall reflect the following:
7.28.1.1. Test mix
7.28.1.2. QC Ultra Low
7.28.1.3. Standard 1
7.28.1.4. Standard 2
7.28.1.5. Case Specimens
7.28.1.6. QC Low
7.28.1.7. QC Ultra Low

7.28.1.8. **Note**: the QC Ultra Low shall be injected at the beginning and at the end of the analytical run (see 9.1.3)

7.29. Place the autosampler vials into the appropriate positions of the autosampler trays of the GC/NPD or GC/MS and have another analyst verify the position of the vials in the autosampler trays, according to each sequence table.

7.30. Initiate the sequence, begin injecting.

7.31. After completion of the sequences on the GC/NPD or GC/MS, transfer the samples to the other instrument (e.g. samples analyzed on GC/NPD are transferred to GC/MS or vice versa) and repeat steps 7.28- 7.30.

7.32. The specimen chromatograms will be reviewed as per the data analysis SOP-054.

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
8.1.5.1.2.1. RTistd = retention time of the internal standard

8.1.6. The HOBSCRN.m (NPD) method is used to calculate raw and adjusted amounts and concentrations for all cases that contain peaks present in the extracted standards. The calculations are generated as follows:

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. Dilution Factor (D)

8.3.1. D = Total volume/Sample volume

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

8.4.1. M = (Vcurve / Vsamp) x D

8.4.1.1. M = Multiplier
8.4.1.2. D = dilution factor
8.4.1.3. Vcurve = matrix volume of calibration curve
8.4.1.4. Vsamp = matrix volume of specimen

8.5. Adjusted Amount

8.5.1. Analyte = {(Aa/Aistd) / (SAa/SAistd)} * M

8.5.2. Analyte = µg of analyte in unknown case (adjusting for dilution)
8.5.3. Aa = area of drug in unknown case
8.5.4. Aistd = area of internal standard in unknown case
8.5.5. SAa = area of drug in standard
8.5.6. SAistd = area of internal standard in standard
8.5.7. D = dilution factor

8.6. Concentration

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

8.6.2. Analyte = µg/mL of analyte in unknown case
8.6.3. Aa = area of drug in unknown case
8.6.4. Aistd = area of internal standard in unknown case
8.6.5. SAa = area of drug in standard
8.6.6. SAistd = area of internal standard in standard
8.6.7. V = volume of sample
8.6.8. 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 (<10% of peak height) from any other peaks in the chromatogram (NPD).
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 QC Ultra Low shall be analyzed as follows:
9.1.3.1. GC/MS – A detailed library report shall be generated for the end of run QC Ultra Low – all target analytes must be present.
9.1.3.2. GC/NPD – Both the pre-run and post-run QC Ultra Low specimens shall be quantitated - all target analytes must be present.
9.1.3.3. If any analytes do not conform to the above criteria or a significant change in peak response between injections is noted, a laboratory supervisor or toxicologist should be notified immediately.
9.1.4. The quality control sample (QC Low) shall have an analytical value as stated on the Standard and Control Worksheet, and shall not deviate ±30% from the expected value.
9.1.4.1. For analytes that don’t conform to the above criteria, only qualitative results may be reported.
9.1.5. The blank shall represent a specimen of “none detected” and should not contain analyte signal above the limit of detection.
9.1.6. The internal standard areas of the samples should be within 50%-200% of the internal standard areas of the calibrators, controls, and blank.

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10. **Validation of Method**

Parameter	**Desired Limit**	**Result**
Bias at decision point	≤ 30%	All analytes evaluated displayed Bias of <30% with the exception of oxycodone. However, oxycodone is present for the sole purpose of helping to determine instrument performance and is not reported from this assay.
Precision at decision point	%CV must not exceed 20%	All analytes evaluated displayed %CV of ≤20%
Calibration model	One point calibration with a positive control at decision point and another at 1/2 decision point.	One point calibration with a positive control at decision point and another at 1/2 decision point (used for evaluation of extraction and instrument sensitivity of low level analytes).
Carryover	No analytes present in blank control injected immediately following 3X calibrator level.	No analytes were detected following injection of calibration standards. In any case specimen in which an analyte peak is greater than 3X that of IS, the following specimen will be evaluated for the presence of possible carryover.
Interference Studies	IS (Alphaprodine) recoveries in fortified blank blood specimens (10) shall exhibit IS recoveries 50-~~150~~ 200% of calibrators and control values.	Matrix interference will be determine on a per specimen basis - Data sources are full scan MS and NPD - detection of unknown analytes is desirable for the purpose of this assay. IS (Alphaprodine) recoveries that are outside 50-~~150~~ 200% of calibrators and control values will be investigated.
LOQ (Set to low QC (QC1) calculated values) - (See Standard and control worksheet).	Must be 0.05 - 0.25 (analyte dependant) ug/mL	Analytes that fall below LOQ to be reported as "less than (LOQ)" those that calculate above LOQ will be Confirmed and Quantitated another assay.
LOD	All peaks must be visible and integratable -	Experimentally determined to be <1/4 of LOQ levels.

10.1.

11. Reporting

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

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

11.3. For compounds detected in other types of specimens (blood, tissue, etc.), report as follows:

11.3.1. Do not report:

11.3.1.1. Analytes or classes of analytes (cocaine, benzodiazepines, opiates/opioids, etc.) that are targeted in the LC Screen or are considered acidic/neutral.

11.3.1.2. Analytes that are detected but exhibit low recovery and/or poor mass spectral match – (shall be labeled as c/w (consistent with) + analyte name on both GC/MS and NPD data).

11.3.2. Report the following drugs qualitatively ("Present"):

11.3.2.1. Nicotine, lidocaine, levamisole, and atropine.

11.3.2.2. Analytes (basic) not represented in the calibration standards.

11.3.3. For analytes that are represented in the calibration standards (see QC "Matrix" tab of the Std & Ctrl Worksheet) that have passed QC criteria (See 9.1.4):

11.3.3.1. If drug concentration is < the low QC level – report as "Less Than (Low QC level)".

11.3.3.2. If drug concentration is > the low QC level – do not report – the reviewing toxicologist will determine whether a confirmation analysis will be ordered.

11.3.3.2.1. **Exception: The limit of quantitation of Amphetamine and Methamphetamine has been raised to 0.25 mg/L (replacing the low QC concentration of 0.125 mg/L). Only cases with amphetamine and or methamphetamine concentrations ≥ 0.25 mg/L shall be quantitated, all others will be reported as "Less Than 0.25 mg/L".**

11.4. If an analyte has failed QC criteria (See 9.1.4), report qualitatively ("Present").

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

11.5.1. "**Organic Bases – None Detected**" - If no drugs are detected

11.5.2. "**Other Organic Bases – None Detected**" - If **all** detected drugs are being reported, and/or LC Screen analytes are present.

11.5.3. "**Other Organic Bases – Present**" - If only some of the analytes detected are being reported due to reasons listed above.

11.5.4. **"Organic Bases - Present" -** If **all** compounds detected are present in the calibration standard **and** quantitate greater than the Low QC **and** LC Screen analytes are not detected.

12. **Load Assignment Packet Preparation**

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

12.1.1. Load assignment sheets, followed by any additional notes to file pertaining to load.
12.1.2. Load specimen sheet.
12.1.3. Chain of Custody.
12.1.4. Standard and Control Worksheet.
12.1.5. GC/NPD and GC/MS Running Sequences.
12.1.6. GC/NPD and GC/MS Testmix chromatograms.
12.1.7. GC/NPD and GC/MS Method Calibration.
12.1.8. Data Analysis Printouts with current area and/or ion ratios for standards 1 and 2.
12.1.9. GC/NPD and GC/MS data for Standard 1, Standard 2, Blank, QC1 (low) and, QC2 (ultra-low).

12.1.10. GC/NPD and GS/MS data for assigned specimens.

12.1.11. The Load Checklist should be initialed and dated to acknowledge completion of load.

13. **References**

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

13.2. Winecker RE: “Quantification of Antidepressants using Gas Chromatography Mass Spectrometry.” *Clinical Applications of Mass Spectrometry*, Hammett-Stabler CH and Garg U, eds. Clifton, NJ: Humana Press, 2010. (pp. 45-56).

13.3. Foerster EH, Hatchett D and Garriott JC. A rapid, comprehensive screening procedure for basic drugs in blood or tissue by gas chromatography. J. Anal Toxicol 1978. 2:50-55.