SOP 202 - Organic Base Quantification by Liquid/Liquid Extraction and analysis by GC/MS and/or NPD.

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North Carolina Office of the Chief Medical Examiner Toxicology Laboratory	**Revision:**	**Revision Date/Initials:**
	8.1.5 – 8.1.8 – Deleted	REW - 04/11/2015
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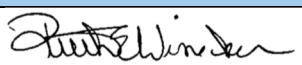
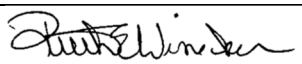
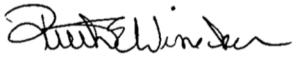
	10.1 – Added validation info.	MSF – 09/09/2015
	9.1.9 – Added reference to QA Manual (Cal point exclusion)	MSF – 04/04/2016
	4 – Added instructions for preparing drug standards	MSF – 05/24/2017
	6 – Updated instrument methods	MSF – 06/05/2017

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

SOP 202 - Organic Base Quantification by Liquid/Liquid Extraction and analysis by GC/MS and/or NPD.

1. **Principle of Assay**

    1.1. This method is designed to quantitate basic drugs previously identified in blood, urine, and tissue specimens by GC-NPD and/or GC/MS. The drugs are extracted from their biological matrix by liquid-liquid extraction and their concentrations calculated via linear regression of calibrators and relative area ratios between the unknown analyte and the internal standard.

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).

        2.2.1. *For non-typical matrices, an additional 0.5mL or 1.0mL aliquot - matrix appropriate - shall be taken (volume permitting), spiked with QC (target analyte), and analyzed to help to identify any matrix effects. (See Non-Matched Matrix Protocol section of the QA/QC manual).

Organic Base Quantification

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
Tissue homogenate	0.5g / 0.05g

        2.2.2. Table 1

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. Drug free blood, urine, and/or liver homogenate (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 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. 3:1 n-Butyl Chloride**:** Ethyl Ether

4.4.1. Into a clean, empty, 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. Pipettes, air displacement 10-100 µL, calibrated
5.4. Pipettes, positive displacement 100-1000 µL, calibrated
5.5. Pipettes, air displacement 100-1000µL, calibrated
5.6. Pipettes, 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. Liner less polypropylene caps (15-415 & 13-415 thread size)

5.12. 2mL GC Autosampler vials
5.13. 0.2mL polyspring inserts
5.14. Crimp caps for 2mL GC Autosampler vials
5.15. Autosampler crimp cap crimper

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. Instrument Methods: "13SPLITOB", "14SPLITOB", "15SPLITOB".
         6.1.3.2. Click here for GC/MS parameters
      6.1.4. Agilent 6890 Gas Chromatograph with Nitrogen Phosphorous Detector (GC/NPD); or equivalent.
         6.1.4.1. Instrument Method: (NPD02, NPD03, NPD04) – "HOBSCREEN"
         6.1.4.2. Click here for GC/NPD parameters

7. **Procedure**

7.1. Select Standard and Control Worksheet(s) that includes all of the analytes to be quantitated.

7.2. Prepare a colored tape label for each standard, control, and specimen to be placed on 16x125mm screw-topped test tubes.

7.3. To the appropriate number of 16X125 screw-topped test tubes, add appropriate amount of internal standard solution. See Standard and Control Worksheet(s)

7.4. Add the appropriate amount of calibration standard to the tubes labeled as standards as outlined on the Standard and Control Worksheet(s).

7.5. Add the appropriate amount of positive control standard(s) to the tube(s) labeled as "QC" as outlined on the Standard and Control Worksheet.

7.6. Pipette 1 mL of blank blood into all standard, blank, and QC samples. Include a urine blank/control (1.0 mL) and/or a liver homogenate blank/control (1.0 g) if there are urine/liver specimens in load.

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

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

7.9. Add 7 mL 3:1 n-butyl chloride/ethyl ether mixture to each tube. Cap each tube and shake vigorously for 3 minutes, or place on rotator for 15 minutes.

7.10. Centrifuge for 10 minutes at 2000 x g.

7.10.1. 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.

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

7.12. Add 2.5 mL of 2N sulfuric acid. 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. Cap each tube and shake vigorously for 3 minutes, or place on rotator for 15 minutes.

7.16. Centrifuge for 5 minutes at 2000 x g.

7.17. Aspirate the top hexane layer to waste.

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

7.19. Vortex for 10 seconds. Transfer each sample to a 5 mL screw-top conical tube.

7.20. Add 100µL n-butyl acetate to each sample with a repeat pipetter.

7.21. Vortex for 20 seconds and centrifuge for 5 minutes at 2000 x g.

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

7.23. Centrifuge at 2000 x g for 5 minutes.

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

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

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

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

7.27. Initiate the sequence, begin injecting.

7.28. The specimen chromatograms will be reviewed 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

8.2. The splitob.m (GC/MS) and HOBSCRN.m (NPD) methods are 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:

8.2.1.1. A linear regression resulting from the drug calibrators is used to quantitate the drug amount in the case.

8.2.1.2. The area of the analyte divided by the area of the internal standard is used in the resulting formula of the calibration curve.

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 only).

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. Controls must calculate within ± 20% of the target 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 shall fall within 50%-200% of the averaged internal standard areas of the calibrators and controls.

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 $R2 \geq 0.992$

9.1.8. The calibrators shall not deviate from target concentration ± 20

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

10. **Validation of Method**

10.1. See "SOP 202 – Organic Base Quantification Validation Summary" located in S:\toxicology\QAQC\SOP

11. **Reporting**

11.1. The percent difference of duplicate analysis for an analyte, when both measurements fall within the linear range of the assay, must be less than or equal to 25% (see Max/Min in Calculations section)

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

Reporting Duplicates

- Dilution factors of 1 and 5 (or other)

Dil / Scenario	1	5	REPORT
A	In curve	In curve	Average
B	In curve	BQL	"In" value
C	AQL	In curve	"In" value
D	In curve	ND (should be in)	Repeat
E	AQL/BQL	AQL/BQL	Less than/Greater than
F	BQL	ND	<LOQ
G	In curve	ND (should be BQL)	"In" value

11.2.1.

11.2.1.1. In Curve = Measured concentration (pre-multiplier) falls within the calibration range.

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

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

11.2.1.4. ND = None Detected

11.3. Averaging reportable values

11.3.1. Results for duplicate analysis (both falling within linear range of assay) shall be truncated prior to averaging.

11.3.2. Enter calculated concentration for each specimen into toxlog.

11.4. Significant figures

11.4.1. Concentrations are truncated and reported with two significant figures in mg/L (mg/kg if aliquot weighed) and a maximum of 3 decimal places e.g. 0.009 mg/L (kg).

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. Aliquot Chain of Custody
12.1.5. Standard and Control Worksheet.
12.1.6. GC/NPD and/or GC/MS Instrument Sequences.
12.1.7. GC/NPD and/or GC/MS Method Calibration.
12.1.8. GC/NPD and/or GS/MS data for calibrators, controls, and assigned specimens.
12.1.9. The Load Checklist should be initialed and dated to acknowledge completion of load.

13. **References**

13.1. Levine, Barry. Principles of Forensic Toxicology. 2nd ed. Washington, D.C.: American Association for Clinical Chemistry, 1999. pp 207-225

13.2. Winecker RE: Quantification of Antidepressants using Gas Chromatography Mass Spectrometry. In: Clinical Applications of Mass Spectrometry, Hammet-Stabler CH and Garg U, eds. Humana Press, Clifton, NJ. 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.