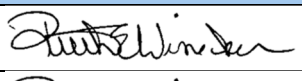
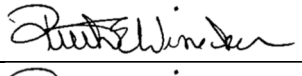
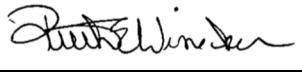
SOP 210 – GHB Extraction using SPE for Quantification by Gas Chromatography/Mass Spectrometry

Table of Contents

SOP Name: GHB Extraction using SPE for Quantification by Gas Chromatography/Mass Spectrometry		SOP #: 210
	Revision:	**Revision Date/Initials:**
North Carolina Office of the Chief Medical Examiner Toxicology Laboratory	9.1.2 – RT acceptance range updated 9.1.5 – IS recovery acceptance range updated 9.1.6 & 9.1.7 – Calibrator acceptance criteria updated	MSF – 5/12/2015
	7.3.1 – Updated cross-reference link	MSF – 03/03/2016
	6.4.1 – Updated Target Ions 9.1.2 – Updated RT window parameters 9.1.8 – Updated ion ratio target origin	MSF – 03/15/2016
	1. – Intro Updated (SCBF) 13. – References Updated (SCBF)	MSF – 10/31/2016

Approving Authority Name	Approving Authority Signature	Approval Date
Ruth E. Winecker, Ph.D.		04/15/2015
Ruth E. Winecker, Ph.D.		06/19/2016
Ruth E. Winecker, Ph.D.		12/07/2017

SOP 210 – GHB Extraction using SPE for Quantification by Gas Chromatography/Mass Spectrometry

1. **Principle of Assay**

1.1. This method is designed to detect the presence of gamma-hydroxybutyrate (4-hydroxybutyrate, GHB) in whole blood, serum, urine, or vitreous by solid phase extraction, followed by derivatization, and analysis by gas chromatography/mass spectrometry.

1.2. GHB is a small, polar compound not easily isolated by conventional methodology. United Chemical Technologies (UCT) has developed a solid phase extraction method that permits the direct isolation of GHB without conversion to gamma-butyrolactone (GBL). This method is a modified version of the UCT procedure.

1.3. Though often linked to drug-facilitated sexual assault allegations, Gamma-hydroxybutyrate (GHB) is considered a drug of abuse popular with bodybuilders and in the rave community. Doses of 10 mg/kg can cause amnesia, Illicit use typically involves doses of 35 mg/kg, and doses of 50 mg/kg and higher produce anesthesia (13.2). The desired effects are euphoria, reduced inhibitions, sedation and muscle relaxation. GHB and its structural analogs which will metabolize to GHB (GBL, 1,4-BD, GVL, GHV) are eliminated rapidly after ingestion (13.3,13.4).

GHB has a natural presence in the body which has often made interpretation of its concentrations challenging. Endogenous levels occur in both blood and urine which makes the differentiation between endogenous production and exposure problematic (13.2).

While this SOP utilizes a lower quantitative limit of 20 mg/L, a publication looking at endogenous concentrations of GHB has recommended a cut-off of 50 mg/L as a majority of the 71 positive samples cited involved concentrations ranging from 10-40 mg/L (13.2). Large postmortem intervals (PMI), can be especially problematic because endogenous levels continue to increase after death (13.5). When calculated blood concentrations are within the reportable range of this assay, the totality of the investigation should be considered with proper communication occurring with the pathologists and the medical examiners on the case.

2. **Specimens**

2.1. This procedure is applicable to urine, blood, serum, vitreous, and plasma specimens. A duplicate sample size of 0.2mL and 0.1mL is generally employed (unless a further dilution is required) so that the concentration of the specimens falls within the calibration range.

3. **Reagents and Materials (HPLC grade)**

3.1. 0.1 M Phosphate buffer, pH 6.0

3.2. Acetone

3.3. Methanol
3.4. Ethyl acetate
3.5. BSTFA + 1% TMCS
3.6. Deionized water
3.7. Ammonium hydroxide
3.8. Blood bank blood

4. **Standards, Controls, and Solutions**
   4.1. Prepare Calibration, IS, and Control standards, as needed, according to SOP 010.

   4.2. 100mM Phosphate Buffer pH 6.0

      4.2.1. To a newly opened 4L bottle of DI H2O, add 48.8 grams of potassium phosphate (KH2PO4) and 1.64 grams sodium hydroxide (NaOH). Swirl until dissolved. Check pH. If necessary, adjust with NaOH or phosphoric acid (H3PO4) to a final pH of 5.5-6.5.

   4.3. Column Elution Solution (make fresh daily)
      4.3.1. To a 100 mL graduated cylinder add 1 mL of concentrated ammonium hydroxide, and fill to the 100 mL with methanol.

5. **Equipment and Special Supplies**
   5.1. Test tubes, 16 x 100mm (or equivalent)
   5.2. Centrifuge 2000 x g
   5.3. Vortex mixer
   5.4. Nitrogen evaporator
   5.5. Positive Pressure Extraction Manifold
   5.6. UCT Clean Screen® GHB extraction columns (ZSGHB020)
   5.7. LC autosampler vials, 12 x 32 mm
   5.8. Polyspring inserts, 5 mm O.D.

6. **Instrumentation**
   6.1. Detector .....Mass Selective Detector
   6.2. Column .....….HP-5MS, HP-1MS, 15m x 0.25µm x 0.25 mm, or equivalent

   6.3. Instrumental Parameters: Agilent 6(5)890 GC/5973(2) EI-MS
      6.3.1. Injection: 1µL in Split Mode (20:1)
      6.3.2. Inlet Temperature: 275°C
      6.3.3. Carrier: Helium
      6.3.4. Initial Flow: 1.5 mL/minute (or equivalent)
      6.3.5. Transfer Line: 280°C
      6.3.6. Oven temperature programming:

6.3.6.1.	Equilibration Time:	30sec.
6.3.6.2.	Initial Temp:	60°C
6.3.6.3.	Initial temp hold:	1 min.
6.3.6.4.	Ramp 1:	35°C/min.
6.3.6.5.	Ramp 1 Hold:	300°C
6.3.6.6.	Total Run Time:	8.86 min.

6.3.7. Inlet Parameters

6.3.7.1.	Mode:	Split
6.3.7.2.	Split Ratio:	20:1
6.3.7.3.	Split Flow:	30.0mL/min.
6.3.7.4.	Temperature:	275°C
6.3.7.5.	Injection Volume:	1µL
6.3.7.6.	Column Flow:	1.5mL/min (Constant Flow)

6.4. GHB Ions (TMS Derivative Form)

6.4.1. Group 1 (Dwell 20 ms):

6.4.1.1. GHB-d6 (**239** 206)

6.4.1.2. GHB (**233** 117 204)

6.4.1.3. **Note:** The ion used for quantification of each analyte is listed first and bolded.

7. Procedure

7.1. Prepare Samples

7.1.1. Prepare a colored tape label for each standard, blank, control, and specimen to be placed on 13x100 mm test tubes.

7.1.2. Add the appropriate quantity (according to the Standard and Control Worksheet) of Deuterated GHB Internal Standard to all the tubes.

7.1.3. Add the appropriate quantity (according to the Standard and Control Worksheet) of GHB calibrator and control standards 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".

7.1.4. Add 200 µL of negative blood bank blood to all standards, controls and blanks.

7.1.5. Add appropriate predetermined amount of unknown specimen to appropriately labeled tube. (See Specimen section).

7.1.6. Add at 1 mL **acetone** to each tube and vortex for 20 seconds.

7.1.7. Centrifuge for 10 minutes at 2000x *g*.

7.1.8. Decant the top acetone layer into clean, appropriately labeled 13x125 mm test tube.

7.1.9. Place in nitrogen evaporator, and evaporate at 60° C to dryness.

7.1.10. Reconstitute dried extracts with at 200µL of Phosphate buffer (pH 6.0) and vortex.

7.2. Prepare SPE Columns:

7.2.1. Load the appropriate number of labeled, new Clean Screen GHB extraction columns (ZSGHB020) for each sample onto a positive pressure extraction manifold.

7.2.2. Introduce 2 mL of methanol into columns and aspirate completely under 2 PSI (positive pressure).

7.2.3. Introduce 2 mL of Di Water into columns and aspirate under 2 PSI (positive pressure).

7.2.4. Introduce 2 mL of Phosphate buffer, pH 6.0 into columns and aspirate under 2 PSI (positive pressure).

7.3. Apply Specimens

7.3.1. Introduce specimens into columns with transfer pipettes (tips changed between samples to prevent cross contamination) and aspirate *slowly* (gravity flow). Save test tubes for elution of drug (7.3.4).

7.3.2. Dry column under 80 PSI positive pressure for approximately 1 minute.

7.3.3. Prepare extraction manifold for specimen collect ion. Place appropriately labeled 16 X 100mm tubes into collection position verifying that SPE tips are securely inside collection tubes.

7.3.4. Introduce 2 mL column elution solution into *original test tube*, vortex for 5 seconds, and decant onto solid-phase column, and collect extract.

7.3.5. Dispose of SPE waste in the appropriate hazardous waste containers.

7.4. Derivatization of samples:

7.4.1. Evaporate each specimen to dryness in a nitrogen evaporation apparatus at 70°C.

7.4.2. Reconstitute each sample with 75 µL **ethyl acetate** and 75 µL of derivatizing reagent (**BSTFA + 1% TMCS**), and vortex. Heating is not required to drive reaction to completion.

7.4.3. Transfer to labeled autosampler vials and inject on the GC/MS.

8. **Calculations**

8.1. Quantification

8.1.1. The GCMS method 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:

8.1.2.1.1. Response Ratio:

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

8.1.2.1.1.2. Response Ratio = QN_a / QN_{istd}

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

8.1.2.1.1.4. 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

8.8. Qualifier Ion Ratios
8.8.1.1.1. Ratio 1 = QL_1/QN

8.8.2. QL_1 = response of the quantifying product ion
8.8.3. QN = response of the qualifying product ion

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.

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. The blank shall represent a specimen of "none detected" and should not contain analyte signal with appropriate ion ratios above 10% of the low standard.

9.1.5. The internal standard areas of the samples shall not deviate more than 50-200% from the average internal standard areas of the calibrators.

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

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

9.1.8. Calculated ion ratios shall not deviate more than 20% to that of calibration level 4.

9.1.9. Results must fall within the linear range of the assay. Results above or below the linear range can only be reported as "less than" the low control or "greater than" the highest control.

9.1.10. Results are reported with two significant figures. All results will be truncated to the nearest significant figure.

10. **Validation of Method**
    10.1. Pending

11. **Reporting**
    11.1. The percent difference of duplicate analysis for an analyte 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 2 (or other)**

Dil / Scenario	1	2	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	ND
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 rang

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 calibration curve) 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/dL.

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. Data summary
12.1.4. Chain of Custody.
12.1.5. Standard Sheet.
12.1.6. GC/MS Method and Calibration Report(s).
12.1.7. Running Sequences.
12.1.8. Chromatograms and results for calibrators, controls and specimens
12.1.9. 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.

13. References

13.1. Baselt, Randall C. Disposition of Toxic Drugs and Chemicals in Man. Foster City, CA: Chemical Toxicology Institute, 2000. 406-08. Print.

13.2. Kintz, P., Villain, M, Cirimele, V., Ludes, B. GHB in postmortem toxicology discrimination between endogenous production from exposure using multiple specimens. *Forensic Science International*. 143 (2004) 177-181.

13.3. Elian, A.A. Determination of endogenous gamma-hydroxybutyric acid (GHB) levels in antemortem urine and blood. *Forensic Science International*. 128 (2002) 120-122.

13.4. National Drug Intelligence Center Information Bulletin: GHB Analogs: GBL, BD, GHV, and GVL. https://www.justice.gov/archive/ndic/pubs1/1621/index.htm accessed on October 31, 2016.

13.5. Moriay, F., Hashimoto, Y. Endogenous gamma-hydroxybutyric acid levels in postmortem blood. *Legal Medicine*. 6 (2004) 47-51.