Liquid – Liquid Extraction of Gamma Hydroxybutyric Acid (GHB) in Blood and Urine for GC-MS analysis

1.0 Purpose - This procedure specifies the required elements for the extraction of GHB from blood, urine and other fluids.

2.0 Scope – This procedure applies to extractions of GHB performed in the Toxicology Units of the State Crime Laboratory.

3.0 Definitions

- **Quality control (QC) check** – Periodic confirmation of the reliability of equipment, instrumentation, and/or reagents.

4.0 Equipment, Materials and Reagents

4.1 Equipment

- Gas Chromatograph- Mass Spectrometer
- Class A Volumetric Flasks
- Mechanical pipettes
- Centrifuge
- Zymark TurboVap LV

4.2 Materials

- Test tubes (16 x 125, 13 x 100, 12 x 75)
- Test tube caps or stoppers
- Vortexer
- Test tube rocker
- Pipet tips
- Pasteur pipets

4.3 Reagents

- Deionized water

4.4 Commercial Reagents

- Sulfuric acid, ACS grade
- Methanol, ACS grade
- Ethyl acetate, ACS grade
- Nitrogen

4.5 Primary Reference Standards

- Gamma Hydroxybutyric Acid, Na (NaGHB)
- Gamma Hydroxybutyric Acid-D_6, Na (NaGHB-D_6)

4.6 Critical Reagents

- Negative Blood
- Negative Urine
- Bis(trimethylsilyl)trifluoroacetamide with 1 % trimethylchlorosilane (BSTFA w/1 % TMCS)

4.7 Prepared Reagents - Prepared reagents may be prepared in any amount provided that the component ratios are kept constant.

4.7.1 0.1 N Sulfuric Acid

4.7.1.1 Add 280 µL concentrated sulfuric acid to approximately 75 mL of deionized water in a 100 mL volumetric flask.

4.7.1.2 Mix and dilute to volume with deionized water.

4.7.1.3 Lot Number: Eight digit format year/month/day/0.1N_H2SO4/initials of preparer.

4.7.1.3.1 Example: 201012310.1N_H2SO4XXX

4.7.1.4 Expiration date**:** Three years.

4.7.1.5 Refrigerate.

4.7.1.6 QC Check: Tests acidic to pH or litmus paper.

4.7.2 GHB Internal Standard Solution

4.7.2.1 Prepare a solution that contains 100 micrograms of NaGHB-D_6/mL in methanol.

4.7.2.1.1 Example: Add 5.0 mL of 1.0 mg/mL NaGHB-D6 to a 50 mL volumetric flask. Mix and dilute to volume with methanol.

4.7.2.2 Lot number: Eight digit format year/month/day/GHBIS/initials of preparer.

4.7.2.2.1 Example: 20101231GHBISXXX

4.7.2.3 Expiration: One year.

4.7.2.4 Refrigerate.

4.7.2.5 QC check: Successful calibration (see **5.1**).

4.7.3 GHB Stock Calibration Solution, purchased or prepared

4.7.3.1 Prepare a solution that contains 1.0 mg/mL of GHB in methanol.

4.7.3.1.1 Example: Quantitatively transfer 121 mg of GHB Sodium to a 100 mL volumetric flask and dilute to volume with methanol.

4.7.3.2 Lot number: Eight digit format year/month/day/GHBStockCal/initials of preparer.

4.7.3.2.1 Example: 20101231GHBStockCalXXX

4.7.3.3 Expiration: One year.

4.7.3.4 Refrigerate.

4.7.3.5 QC check: Successful calibration (see **5.1**).

4.7.4 100 µg/mL GHB Calibration Solution, purchased or prepared

4.7.4.1 Prepare a dilution of the GHB Stock Calibration Solution from **4.7.3** that contains 100 µg/mL GHB in methanol.

4.7.4.1.1 Example: Add 1.0 mL of GHB Stock Calibration Solution to a 10 mL volumetric flask and dilute to volume with methanol.

4.7.4.2 Lot number: Eight digit format year/month/day/GHBCal100/initials of preparer.

4.7.4.2.1 Example: 20101231GHBCal100XXX

4.7.4.3 Expiration: One year.

4.7.4.4 Refrigerate.

4.7.4.5 QC check: Successful calibration (see **5.1**).

4.7.5 50 µg/mL GHB Calibration Solution, purchased or prepared

4.7.5.1 Prepare a dilution of the GHB Stock Calibration Solution from **4.7.3** that contains 50 µg/mL GHB in methanol.

4.7.5.1.1 Example: Add 500 µL of GHB Stock Calibration Solution to a 10 mL volumetric flask and dilute to volume with methanol.

4.7.5.2 Lot number: Eight digit format year/month/day/GHBCal50/initials of preparer.

4.7.5.2.1 Example: 20101231GHBCal50XXX

4.7.5.3 Expiration: One year.

4.7.5.4 Refrigerate.

4.7.5.5 QC check: Successful calibration (see **5.1**).

4.7.6 10 µg/mL GHB Calibration Solution, purchased or prepared

4.7.6.1 Prepare a dilution of the GHB Stock Calibration Solution from **4.7.3** that contains 10 µg/mL GHB in methanol.

4.7.6.1.1 Example: Add 100 µL of GHB Stock Calibration Solution to a 10 mL volumetric flask and dilute to volume with methanol.

4.7.6.2 Lot number: Eight digit format year/month/day/GHBCal10/initials of preparer.

4.7.6.2.1 Example: 20101231GHBCal10XXX

4.7.6.3 Expiration: One year.

4.7.6.4 Refrigerate.

4.7.6.5 QC check: Successful calibration (see **5.1**).

4.7.7 GHB Stock Verification Solution, purchased or prepared

4.7.7.1 Prepare or purchase a solution that contains 1.0 mg/mL of GHB in methanol.

4.7.7.1.1 Example: Quantitatively transfer 121 mg of GHB Sodium to a 100 mL volumetric flask and dilute to volume with methanol.

4.7.7.2 Lot number: Eight digit format year/month/day/GHBStockVer/initials of preparer.

4.7.7.2.1 Example: 20101231GHBStockVerXXX

4.7.7.3 Expiration: One year.

4.7.7.4 Refrigerate.

4.7.7.5 QC check: Successful calibration (see **5.1**).

4.7.8 100 µg/mL GHB Verification Solution, purchased or prepared

4.7.8.1 Prepare a dilution of the GHB Stock Verification Solution from **4.7.7** which contains 100 µg/mL GHB in methanol.

4.7.8.1.1 Example: Add 1.0 mL of GHB Stock Verification Solution to a 10 mL volumetric flask and dilute to volume with methanol.

4.7.8.2 Lot number: Eight digit format year/month/day/GHBVer100/initials of preparer.

4.7.8.2.1 Example: 20101231GHBVer100XXX

4.7.8.3 Expiration: One year.

4.7.8.4 Refrigerate.

4.7.8.5 QC check: Successful calibration (see **5.1**).

4.7.9 50 µg/mL GHB Verification Solution, purchased or prepared

4.7.9.1 Prepare a dilution of the GHB Stock Verification Solution from **4.7.7** which contains 50 µg/mL GHB in methanol.

4.7.9.1.1 Example: Add 500 µl of GHB Stock Verification Solution to a 10 mL volumetric flask and dilute to volume with methanol.

4.7.9.2 Lot number: Eight digit format year/month/day/GHBVer50/initials of preparer.

4.7.9.2.1 Example: 20101231GHBVer50XXX

4.7.9.3 Expiration: One year.

4.7.9.4 Refrigerate.

4.7.9.5 QC check: Successful calibration (see **5.1**).

4.7.10 10 µg/mL GHB Verification Solution, purchased or prepared

4.7.10.1 Prepare a dilution of the GHB Stock Verification Solution from **4.7.7** which contains 10 µg/mL GHB in methanol.

4.7.10.1.1 Example: Add 100 µl of GHB Stock Verification Solution to a 10 mL volumetric flask and dilute to volume with methanol.

4.7.10.2 Lot number: Eight digit format year/month/day/GHBVer10/initials of preparer.

4.7.10.2.1 Example: 20101231GHBVer10XXX

4.7.10.3 Expiration: One year.

4.7.10.4 Refrigerate.

4.7.10.5 QC check: Successful calibration (see **5.1**).

5.0 Procedure

5.1 Calibration

5.1.1 The Toxicology GC-MS Key Operator or designee shall calibrate the GC-MS GHB Blood and GHB Urine methods upon preparation of a new lot of GHB Internal Standard solution and after instrument maintenance that may affect the calibration as determined by the Toxicology GC-MS Key Operator.

5.1.2 GHB Blood Calibration Standards

5.1.2.1 Prepare each of the following in duplicate using negative blood.

5.1.2.1.1 Negative GHB Blood Calibration Standard - label a clean test tube.

5.1.2.1.2 5.0 µg/mL GHB Blood Calibration Standard - add 100 µL of the 50 µg/mL GHB calibration solution to a clean test tube.

5.1.2.1.3 10 µg/mL GHB Blood Calibration Standard - add 200 µL of the 50 µg/mL GHB calibration solution to a clean test tube.

5.1.2.1.4 25 µg/mL GHB Blood Calibration Standard - add 250 µL of the 100 µg/mL GHB calibration solution to a clean test tube.

5.1.2.1.5 50 µg/mL GHB Blood Calibration Standard - add 500 µL of the 100 µg/mL GHB calibration solution to a clean test tube.

5.1.2.1.6 100 µg/mL GHB Blood Calibration Standard - add 1.0 mL of the 100 µg/mL GHB calibration solution to a clean test tube.

5.1.2.2 Prepare as directed in **5.5**.

5.1.3 GHB Blood Verifiers

5.1.3.1 Prepare each of the following using negative blood.

5.1.3.1.1 Negative GHB Blood Verifier - use a clean test tube.

5.1.3.1.2 10 µg/mL GHB Blood Verifier - add 200 µL of the 50 µg/mL GHB calibration solution to a clean test tube.

5.1.3.1.3 25 µg/mL GHB Blood Verifier - add 250 µL of the 100 µg/mL GHB calibration solution to a clean test tube.

5.1.3.1.4 100 µg/mL GHB Blood Verifier - add 1.0 mL of the 100 µg/mL GHB calibration solution to a clean test tube.

5.1.3.2 Prepare as directed in **5.5**.

5.1.4 GHB Urine Calibration Standards

5.1.4.1 Prepare each of the following in duplicate using negative urine.

5.1.4.1.1 Negative GHB Urine Calibration Standard – label a clean test tube.

5.1.4.1.2 5.0 µg/mL GHB Urine Calibration Standard – add 100 µL of the 10 µg/mL GHB calibration solution to a clean test tube.

5.1.4.1.3 10 µg/mL GHB Urine Calibration Standard – add 200 µL of the 10 µg/mL GHB calibration solution to a clean test tube.

5.1.4.1.4 25 µg/mL GHB Urine Calibration Standard – add 100 µL of the 50 µg/mL GHB calibration solution to a clean test tube.

5.1.4.1.5 50 µg/mL GHB Urine Calibration Standard – add 200 µL of the 50 µg/mL GHB calibration solution to a clean test tube.

5.1.4.1.6 100 µg/mL GHB Urine Calibration Standard – add 400 µL of the 50 µg/mL GHB calibration solution to a clean test tube.

5.1.4.1.7 200 µg/mL GHB Urine Calibration Standard – add 400 µL of the 100 µg/mL GHB calibration solution to a clean test tube.

5.1.4.2 Prepare as directed in **5.6**.

5.1.5 GHB Urine Verifiers

5.1.5.1 Prepare each of the following using negative urine.

5.1.5.1.1 Negative GHB Urine Verifier - use a clean test tube

5.1.5.1.2 10 µg/mL GHB Urine Verifier – add 200 µL of the 10 µg/mL GHB verification solution to a clean test tube.

5.1.5.1.3 50 µg/mL GHB Urine Verifier – add 200 µL of the 50 µg/mL GHB verification solution to a clean test tube.

5.1.5.1.4 200 µg/mL GHB Urine Verifier – add 400 µL of the 100 µg/mL GHB verification solution to a clean test tube.

5.1.5.2 Prepare as directed in **5.6**.

5.1.6 Chromatograph and quantitate the calibration standards on the GC-MS using the GHB Blood GC-MS method for the blood standards and the GHB Urine GC-MS method for the urine standards.

5.1.7 For blood, update the GHB Blood calibration with the blood calibration standards. For urine, update the GHB Urine calibration with the urine calibration standards.

5.1.7.1 The response at each level shall be the response of the calibration standard at that level.

5.1.7.2 For blood, the retention time shall be the average retention time of the 25 µg/mL calibration standard duplicates. For urine, the retention time shall be the average retention time of the 50 µg/mL calibration standard duplicates.

5.1.7.3 For blood, the ion ratios shall be the average of the 25 µg/mL calibration standard duplicates. For urine, the ion ratios shall be the average of the 50 µg/mL calibration standard duplicates.

5.1.8 The calibration curve shall be fitted to a linear model.

5.1.9 The calibration curve shall have a correlation of determination (r^2) of 0.985 or greater. If the calibration has a correlation of determination of less than 0.985, appropriate action (i.e., maintenance or new solution preparation) shall be taken and the calibration repeated.

5.1.10 After each successful calibration, save the data analysis method according to the format "GHB" (insert blood or urine) eight digit format year/month/day.

5.1.11 Calibration Verification

5.1.11.1 Chromatograph the verifiers and quantitate with the corresponding data analysis method from **5.1.7**.

5.1.11.2 The calibration curve shall meet the criteria below. If the calibration does not meet the criteria below, appropriate action (i.e., maintenance or new solution preparation) shall be taken and the calibration and / or calibration verification shall be repeated.

5.1.11.2.1 GHB shall not be identified in the negative verifier.

5.1.11.2.2 The retention times of the GHB and internal standard shall not differ by more than 2.0 % from the target value.

5.1.11.2.3 The qualifier ion ratios of the GHB and internal standard shall be within +/- 20 % of the target value.

5.1.11.2.4 The GHB quantitation results shall be within +/- 20 % of the target value.

5.1.12 The calibration data shall be reviewed by another qualified Forensic Scientist and, if acceptable, approved in the Toxicology Unit section object repository in FA with the name of the data analysis method from **5.1.10**.

5.1.13 The calibration data packet shall include the following:

- Lot number of the GHB calibration solution
- Lot number of the GHB verification solution
- Lot number and expiration date of the GHB internal standard
- Lot number of negative blood or negative urine
- GC-MS sequence table
- GC-MS tune
- GC-MS method
- Quantitation reports for each calibration and verification standard
- Printed calibration table
- GHB r^2 value

5.1.14 Record each calibration in the GC-MS instrument log with the date, lot number of internal standard used, and internal standard expiration date and operator initials.

5.2 Maintenance

5.2.1 Add water to the TurboVap if needed.

5.3 Sampling

5.3.1 Allow all solutions and samples to be analyzed to equilibrate to room temperature.

5.3.2 Ensure that all body fluids are homogenous by shaking and/or vortexing.

5.3.3 If a homogenous sample cannot be obtained, a notation shall be made in the worksheet detailing the condition of the sample and its handling.

5.4 Standards and Controls

5.4.1 Each extraction batch shall contain two positive and two negative controls. If necessary, add additional positive and/or negative controls so that the batch contains at least 10 % controls.

5.4.1.1 The positive and negative controls shall meet the criteria below for each extraction batch. If the positive and negative controls do not meet the criteria below, appropriate action (e.g., maintenance or new solution preparation) shall be taken and the batch shall be extracted again in accordance with the State Crime

Laboratory Procedure for Corrective Action and Non-Conformities. Note any problems in the GC-MS instrument log.

5.4.1.2 Positive control

5.4.1.2.1 The internal standard qualifier ion ratios shall be within +/- 20 % of the target value.

5.4.1.2.2 For each extraction batch, prepare a positive control in duplicate as directed in **5.1.3** for blood or **5.1.5** for urine. The matrix (blood or urine) of the positive control shall match that of the samples being analyzed.

5.4.1.2.3 The qualifier ion ratios of the positive control duplicate that was not used to set the target values shall be within +/- 20 % of the target values.

5.4.1.2.4 The GHB quantitation results shall be within +/- 20 % of the target value.

5.4.1.3 Negative Control

5.4.1.3.1 For each extraction batch prepare a negative control as directed in **5.1.3** for blood or **5.1.5** for urine. The matrix (blood or urine) of the negative control shall match that of the samples being analyzed.

5.4.1.3.2 The internal standard qualifier ion ratios shall be within +/- 20 % of the target values.

5.4.1.3.3 GHB shall not be identified in the negative control. If GHB is present in the negative control, the extraction shall be repeated.

5.5 Blood Sample preparation

5.5.1 In duplicate, add 100 µL GHB internal standard solution to a clean (or previously prepared for calibration standards and verifiers) test tube.

5.5.2 Evaporate to dryness using a TurboVap.

5.5.3 Add 1.0 mL of the blood to be analyzed to the test tube and vortex.

5.5.4 Proceed to **5.7**.

5.6 Urine Sample Preparation

5.6.1 In duplicate, add 40 µl of the GHB internal standard solution to a clean (or previously prepared for calibration standards and verifiers) test tube.

5.6.2 Evaporate to dryness using a TurboVap.

5.6.3 Add 0.2 mL of the urine to be analyzed and 0.8 mL of deionized water to the test tube and vortex.

5.6.4 Proceed to **5.7**.

5.7 Extraction Procedure

5.7.1 Add 250 µL of cold 0.1 N H_2SO_4 and mix/vortex.

5.7.2 Add 4 mL of ethyl acetate and cap the test tube securely.

5.7.3 Place the test tubes on the test tube rocker and allow to mix for 10 minutes.

5.7.4 Centrifuge for 5 minutes.

5.7.5 Transfer the organic layer (upper) to a clean test tube, taking care to not transfer any of the non-organic layer.

5.7.6 To the test tube containing blood or urine, add 4 mL of ethyl acetate and cap the test tube securely.

5.7.7 Repeat steps **5.7.3** and **5.7.4**.

5.7.8 Transfer the organic layer into the test tube that contains the previously transferred organic layer from step **5.7.5**.

5.7.9 Evaporate the collected organic layer to dryness using a TurboVap.

5.7.10 Add 50 µL of BSTFA w/1 % TMCS and cap securely.

5.7.11 Mix and heat for 15 minutes at 80 °C.

5.7.12 Cool to room temperature.

5.7.13 Transfer to an insert in an auto-sampler vial and cap securely.

5.8 Post Extraction Procedure

5.8.1 For blood, chromatograph each duplicate using the GHB Blood GC-MS method as specified in the Toxicology Gas Chromatography/Mass Spectrometry (GC-MS) procedure. For urine, chromatograph each duplicate using the GHB Urine GC-MS method as specified in the Toxicology Gas Chromatography/Mass Spectrometry (GC-MS) procedure. For blood and urine also chromatograph one duplicate using the GHBFS GC-MS method as specified in the Toxicology Gas Chromatography/Mass Spectrometry (GC-MS) procedure. All extraction batches will begin and end with a positive and negative control.

5.8.2 Save the most current calibrated GHB Blood or GHB Urine GC-MS method with the initials of the Scientist and the date added to the end of the method name. The method shall correspond to the lot number of GHB Internal Standard Solution used to prepare the samples.

5.8.3 Use a positive control to replace the qualifier ion ratios and retention times of each component. The updated values are the target values.

5.8.4 Save the updated method.

5.8.5 Quantitate the remaining extracts using the updated GHB Blood or GHB Urine GC-MS data analysis method from **5.8.4**.

5.8.6 The Quality Control data packet shall be reviewed by a Forensic Scientist qualified to perform Liquid-Liquid Extraction of Gamma Hydroxybutyric Acid (GHB) in Blood and Urine for GC-MS Analysis and, if acceptable, approved in the Toxicology Unit section object repository of FA with a file name beginning with "GHBQC" (capitalization optional), followed by eight digit format year/month/day. The name shall have a suffix included to distinguish between blood and urine analyses.

5.8.6.1 Example: GHBQC20121004-blood or GHBQC20121004-urine

5.8.7 The quality control data packet shall include the following:

- Lot number and expiration date of the GHB internal standard solution
- Lot number of the GHB verification solution
- Lot number of negative blood and/or urine
- Lot number of BSTFA with 1% TMCS
- GC-MS Sequence table
- GC-MS tune
- GHB GC-MS method and calibration curves printed at the time of data analysis
- Quantitation reports of the positive control and negative controls

5.8.8 Control Charting

5.8.8.1 Complete the Toxicology Control Chart Form and submit to the Toxicology Technical Leader.

5.9 Identification of GHB

5.9.1 The internal standard area of the quantifier ion shall be greater than 50 % of the average of the area of the quantifier ion of the controls.

5.9.1.1 If the internal standard area is less than 50 %, the quantitation shall not be used. The case will be reanalyzed, sample volume permitting.

5.9.1.2 If there is insufficient sample volume remaining, the data may be reported qualitatively only if the acceptance criteria in **5.9.2**, **5.9.3**, **5.9.4**, and **5.9.5** are met.

5.9.2 The qualifier ion ratios of each duplicate shall be within +/- 20 % of the target value.

5.9.3 The quantifier ion retention time of each duplicate shall not differ from the target value by more than 2.0 percent.

5.9.4 The quantitation result of each duplicate shall be 10 µg/mL or greater.

5.9.5 GHB shall be identified with a full scan mass spectrum comparison to a reference standard in one duplicate. Refer to the Toxicology Unit Toxicology Gas Chromatography - Mass Spectrometry (GC-MS) procedure.

5.10 Reporting

5.10.1 If the positive control fails for GHB, the sample may be reported negative as long as GHB is not present in both case sample duplicates.

5.10.1.1 If GHB is present, the sample shall be re-extracted.

5.10.2 Refer to the Toxicology Unit Toxicology Analysis procedure for reporting of identified and unidentified GHB.

5.11 Record the following in the case record

- Approved GHB GC-MS method calibration data packet
- Quantitation report of the sample
- Approved GHBQC data packet for run

5.12 Calculations

5.12.1 Percent Difference Calculation:
|(standard retention time-analyte retention time)|/(standard retention time) * 100

5.13 Uncertainty of Measurement – N/A

6.0 Limitations

6.1 Samples with GHB concentrations that exceed the upper level of the calibration curve may be reanalyzed after dilution with the proper matrix to bring them within the calibration range or be recorded as "quantitation exceeded the (upper concentration limit) upper limit of calibration."

7.0 Safety

7.1 Refer to the State Crime Laboratory Safety Manual.

8.0 References

Couper, Fiona J. and Barry K. Logan. "Determination of Gamma-Hydroxybutyrate (GHB) in Biological Specimens by Gas Chromatography-Mass Spectrometry." *Journal of Analytical Toxicology*. Vol. 24, (January/February 2000): 1-7.

McCusker, Rachel R., et al. "Analysis of Gamma-Hydroxybutyrate (GHB) in Urine by Gas Chromatography-Mass Spectrometry." *Journal of Analytical Toxicology*. Vol. 23, (September 1999): 301-305.

Baselt, Randall C. and Robert H. Cravey. *Disposition of Toxic Drugs and Chemicals in Man*, Fourth Edition. Chemical Toxicology Institute, Foster City, CA (1995).

9.0 Records

- Calibration data
- Case record
- Toxicology Control Chart Form

10.0 Attachments- N/A

Revision History		
Effective Date	Version Number	Reason
09/17/2012	1	J-13 Conversion to ISO format
10/26/2012	2	5.1.13 - inserted additional requirements into Calibration data packet; 5.1.4.2.4 - removed control chart reference; 5.4.1.3.3 - inserted response to failed negative control; 5.4.2 - moved to Post extraction procedure (5.8) and changed to new control charting form; 5.5.2, 5.6.2, and 5.7.9 - removed dry down temperature added "using TurboVap"; 5.7.4: removed speed setting for centrifuge; 5.8.1 - created reference to appropriate GC-MS procedure and reworded for consistency; 5.8.3 - removed duplicate; 5.8.4 - reworded for clarification; 5.8.6 and 5.8.7 - created requirements and criteria for GHB QC data packet; 5.9.1.2 - included the criteria to be reported under 5.9.1.2; 5.9.3 - clarified which retention time being used; 5.11 - updated calibration data packet language, inserted the QC data packet created in 5.8.6 and 5.8.7; 5.10.1 - inserted criteria for negative reporting; 5.10.2 - inserted unidentified reporting; 5.12 - removed rounding requirement; 6.1 - added reporting language; 9.0 - changed control chart form record title to reflect new control chart form
02/15/2013	3	2.0 - modified for procedure merge 5.1.5.1.1 - corrected matrix 5.1.5.1.4 - inserted matrix 5.1.13 - inserted GC-MS method