1. Introduction

Gamma-hydroxybutyrate, or gamma-hydroxybutyric acid, (GHB) is a central nervous system depressant agent acting as a GABA-receptor agonist. It is endogenous to humans, beef, and some vegetation. In humans it can be produced from succinic semialdehyde by the action of an NADPH-dependent aldo-keto reductase. The compound can be used as a psychotropic agent and has been referred to as a drug-facilitated sexual assault (DFSA) drug. It is categorized as an illegal drug, but can be used to treat conditions such as insomnia, depression, and alcoholism. Endogenous levels of GHB are generally below 1 mg/L, but higher levels may result as a consequence of oxidation of endogenous GABA (gamma-aminobutyric acid). When exogenous ingestion is suspected, forensic toxicological samples (e.g., blood, urine) may contain GHB levels well above 10 mg/L.

2. Scope

This method is primarily intended to be used for the identification and quantitation of GHB within appropriate specimens (e.g., blood, urine). It involves using solid phase extraction (SPE) columns. Derivatization is applied using a siloxyl-containing agent, N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) to allow GHB to be separated and detected by gas chromatography/mass spectrometry (GC/MS).

3. Principle

Blood and urine samples are the two most common type of toxicological specimens for requests involving the detection and quantitation of GHB. Other liquids (e.g., adulterated beverages) may be appropriate samples for GHB testing by this method, but dilution should be considered. Following the addition of a deuterated analog as an internal standard, samples are extracted with acetone coupled with a SPE column. If present within a sample, GHB is collected and taken to dryness, followed by derivatization with BSTFA. The sample is analyzed by GC/MS in the electron-impact mode (EI).

4. Specimens

This procedure typically uses 200 µL of blood, urine. Other liquids may be analyzed, but dilution and variation of the sample volume may be necessary.

5. Equipment/Materials/Reagents

Note: Reagents are ACS grade or better unless otherwise specified

A. GC/MS and associated data station/computer (HP6890/5973 or equivalent)
B. Glass test tubes 16 x100mm (or equivalent)
C. 1.5 mL polypropylene flat top microcentrifuge tubes (or equivalent)
D. Pipette (5-20 µL) with disposable tips (or equivalent)
E. Pipette (20-200 µL) with disposable tips (or equivalent)
F. Pipette (0.1-1.0 mL) with disposable tips (or equivalent)
G. Vortex mixer
H. Micro-vial centrifuge
I. Evaporator with nitrogen (or equivalent)
J. SPE vacuum manifold or positive pressure manifold (or equivalent)
K. Clean Screen® GHB extraction column: (UCT, Inc., Part#: CSGHB203 or equivalent)
L. Heating block
M. Acetone
N. Methanol
O. Ammonium hydroxide, concentrated
P. Ethyl acetate
Q. N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% TMCS (trimethylchlorosilane)
R. DI Water (Distilled or Deionized)
S. Disodium phosphate (Na_2HPO_4)
T. Monosodium phosphate (NaH_2PO_4)
U. pH paper
V. GHB-D_6 sodium salt solution (Cerilliant G-006, 1.0 mg/mL as salt): (or equivalent)
W. GHB sodium salt solution (Cerilliant G-001, 1.0 mg/mL as salt): (or equivalent)
X. Utak GHB dried whole blood control (or equivalent)
Y. Utak GHB dried urine control (or equivalent)
Z. Control blood (Blood Bank or equivalent)
AA. Control Urine (purchased or obtained in-house)

6. Reagent Preparation

Reagent preparation and validation is documented in the Toxicology "Reagent Preparation/Validation Logbook" maintained in the Toxicology Unit.

Phosphate Buffer (~0.1M, pH ~ 6.0):

1. Dissolve 1.70g Na_2HPO_4 and 12.14g NaH_2PO_4 in 800 mL D.I. Water;
2. Dilute to 1000mL using water and mix
3. Using pH paper ensure that the pH is ~6.0

GHB Elution Solvent - (1%(v/v) NH_4OH in MeOH):

1. Combine 1 mL of concentrated ammonium hydroxide with 99 mL of methanol using a volumetric glassware (e.g., graduated cylinder)
2. Mix well

7. Standards & Controls

Preparation of all control solutions is documented in the "Calibrator and Control Preparation Log" (can be maintained in the Toxicology Unit's - wet laboratory area).

Reference Standards:

1. Na-GHB-D_6 Stock Solution (Cerilliant G-006, 1.0 mg/mL as salt) :
   MW (Na-GHB-D_6) = 132.12 MW (GHB-D_6) = 110.13
2. Na-GHB Stock Solution (Cerilliant G-001, 1.0 mg/mL as salt):
   MW (Na-GHB) = 126.09 MW (GHB) = 104.10

Internal Control Solutions:

1. GHB-D_6 Working Internal Solution (100 µg/mL)
   a. Dilute 240µL of Na-GHB-D_6 Stock Solution (1.0 mg/mL) to 2 mL with water*
   b. If stored refrigerated it is stable for at least 1 year
2. GHB Working Internal Solution (100 µg/mL):
   a. Dilute 242µL of Na-GHB Stock Solution (1.0 mg/mL) to 2 mL with water*
   b. If stored refrigerated it is stable for at least 1 year

*Note: MeOH can be substituted for H_2O and Internal Control Solutions can then be stored in a freezer.

External Control Solutions:

1. Blood Control (10 µg/mL GHB)
   a. Reconstitute Utak dried whole blood control with 5 mL water using a volumetric pipette
   b. Cap and let sit 10-15 minutes
   c. Gently swirl 3-4 minutes or mix on rotator until all particles are dissolved into a homogeneous mixture. Swirl gently each time before an aliquot is removed to ensure the solution is homogeneous.

   Note: An alternate to using the Utak dried whole blood product is to use GHB-free whole blood and add an appropriate amount of known GHB to make a 10 µg/mL GHB control blood solution.
2. Urine Control (20 µg/mL GHB and 100 µg/mL GHB)
   a. Reconstitute Utak dried urine controls (20 µg/mL and 100 µg/mL) with 5 mL water using a volumetric pipette
   b. Cap and let sit 10-15 minutes

c. Gently swirl 3-4 minutes or mix on rotator until all particles are dissolved into a homogeneous mixture. Swirl gently each time before an aliquot is removed to ensure the solution is homogeneous.

Note: An alternate to using the Utak dried urine product is to use GHB-free urine and add an appropriate amount of known GHB to make 20 µg/mL and 100 µg/mL GHB urine control solutions.

3. Negative Control Blood: GHB-free human blood, (whole blood or reconstituted from dried blood)
4. Negative Control Urine: GHB-free human urine, (intact urine or reconstituted from dried urine)

Note: Controls should be prepared from reference materials that have a different source (e.g., lot number or supplier) than those used to prepare calibrator solutions.

8. Calibration

Preparation of all control solutions is documented in the "Calibrator and Control Preparation Log" (can be maintained in the Toxicology Unit's - wet laboratory area).

Table 1 shows the concentrations and volumes used for the preparation of calibration solutions in water or other matrix for GHB analysis.

Table 1: Sample calibrator preparation

Calibrator level (µg/mL GHB)	Total Volume	Sample Added	Volume of GHB Working Control Solution (100µg/mL)
0	200 µL	200 µL Urine	0 µL
0	200 µL	200 µL Blood	0 µL
2	200 µL	196 µL Water	4 µL
5	200 µL	190 µL Water	10 µL
10	200 µL	180 µL Water	20 µL
25	200 µL	150 µL Water	50 µL
50	200 µL	100 µL Water	100 µL
100	200 µL	0 µL Water	200 µL

Table 2 shows the concentrations and volumes for controls

Table 2: Sample calibrator preparation

Control level (µg/mL)	Volume	QC used
10	200 µL	Utak GHB 10 whole blood
20	200 µL	Utak GHB 20 urine
100	200 µL	Utak GHB 100 urine

9. Procedure

A. Pipet 200 µL of sample into properly labeled 1.5 mL plastic centrifuge tubes

B. Add 25 µL of GHB-D_6 Working Internal Solution (100 µg/mL) to all tubes

C. Add 1.0 mL of acetone to each tube; cap, and vortex-mix each tube for ~15 seconds

D. Centrifuge all tubes for ~5 minutes

E. Transfer using a pipette (with disposable tip) 800 µL of the top acetone layer into a properly labeled test tube

F. Evaporate the organic phase to dryness using a stream of nitrogen gas at ~50 °C

G. Reconstitute the dried extract with 200 µL of 0.1M phosphate buffer (pH ~ 6.0)

H. Vortex-mix for ~15 seconds

I. Condition the Clean Screen® GHB extraction column**: (CSGHB203 or equivalent)
   1. 1 x 3 mL Methanol
   2. 1 x 3 mL D.I. water
   3. 1 x 1 mL 0.1 M Phosphate buffer (pH ~ 6.0)
   4. Aspirate at 3 inches of Hg or less to prevent sorbent drying

   **Note: If smaller columns are used, adjust volumes accordingly

J. Apply the sample by pipetting (Eppendorf or Rainin) all reconstituted samples to properly labeled SPE columns

K. Elute GHB:
   1. Place clean test tubes into the collecting manifold
   2. Add 1 mL of CH_3OH/NH_4OH (100:1) to each SPE column
   3. Collect the extract

L. Evaporate the extract to dryness at ~60 °C using a stream of dry nitrogen

M. Reconstitution and Derivatization:
   1. Add 100 µL of ethyl acetate

2. Vortex-mix and transfer into GC vials that contain inserts
3. Add 25 µL of BSTFA with 1% TMCS to GC vials, cap, and mix
4. Heat all samples, calibrators, and controls for 10 minutes at ~70 °C in a dry-block incubator
5. Allow solutions within GC vials to cool
6. Analyze 1 µL of solutions using GC/MS

N. Quantitation can be done by using SIM mode mass spectral (MS) data:

Compound	Primary Ion	Secondary	Tertiary
GHB-D_6-di-TMS	239	240	241
GHB-di-TMS	233	234	235

Note: Batches for GHB should follow the format indicated below: Bracket casework with controls at the beginning and end of sequence. For long runs control(s) should be added in the middle of the batch. The following 'Batch Format' should be used:

Calibrators
Blank negative urine control
Blank negative blood control
Positive Control(s) beginning
Solvent blank
Samples
Positive Control(s)

10. Instrument Conditions

The following parameters can be used for this analysis:

A. Gas Chromatograph Parameters

Oven parameters		Inlet and Carrier parameters		Column Parameters	
Initial Temperature	80 °C	Injection Mode	Pulsed splitless	Type	RTX-1
Hold time	1.00 min	Inlet temperature	250 °C	Length	30 m
Ramp rate 1	15 °C /min	Pressure	9.29 psi	Internal dia.	0.25 mm
Temperature 2	100 °C	Pulse pressure	35 psi	Film thickness	0.25 µm
Ramp rate 2	25 °C/min	Pulse time	0.50 min	Flow mode	Constant
Temperature 3	175°C	Purge flow	40.0 mL/min	Flow rate	1.0 mL/min
Ramp rate 3	40 °C/min	Purge time	0.50 min		
Final temperature	325 °C	Total flow	44.0 mL/min		
Post temperature	80 °C	Gas saver	On (helium)		
Total run time	9.08 min	Saver flow	20.0 mL/min		
		Saver time	0.80 min		
		Injection volume	1 µL		

B. Mass Spectrometer Parameter

Ionization mode	Electron Impact		Source temperature	230 °C
Acquisition mode	SIM		Transfer line temperature	280 °C
m/z group	233, 234, 235, 239, 240, 241		Solvent delay	3 min

11. Decision Criteria

The following criteria are used as guidelines in determining the acceptability of the data produced in this assay. In general, compound identification should be based on a comparison of the chromatography and mass spectrometry for the analyte peak of interest with data from a contemporaneously analyzed reference standard or extracted positive control. In most cases all of the following should be met in order to identify GHB within a specimen:

A. Chromatography:
The peak of interest should show good chromatographic fidelity, with reasonable peak shape, width, and resolution.

B. Retention time:
The retention time of the peak should be within ±5 % of the retention time (relative or absolute)

obtained from injection of a reference standard or extracted positive control of GHB.

C. Signal-to-Noise:
To justify the existence of a peak, its baseline signal to peak-to-peak noise ratio should exceed 3. Further, the baseline signal for the peak from the sample of interest should be at least 10 fold greater than that for any observed peak at a similar retention time in a negative control that's being analyzed within the same batch.

D. Mass Spectrometry:
The mass spectrum of the analyte of interest should match that of the appropriate reference standard or an extracted positive control. The ion ratios for both qualitative ions must be within ±20 % of the corresponding ratio of calibrator sample.

E. Quantitations:
Calibration for each batch is done independently. No sample analysis should use a historical calibration curve. Linearity of the calibration curve is demonstrated within each batch, with R^2>0.95 as an acceptable criteria. Quantitation acceptability of the batch is also assessed by matrix specific controls which can be house made or/and commercially obtained. In house GHB controls (10 µg/mL, 25 µg/mL, 100 µg/mL) or commercially available external controls (e.g., UTAK blood 10 µg/mL GHB, urine 20µg/mL GHB to 100 µg/mL GHB) are typically used. Quantitation is calculated based on the primary ion's abundance.

12. Limitations

A. Limit of Detection: 2 µg/mL for GHB
B. Limit of Quantitation: 5 µg/mL for GHB
C. Accuracy and precision: Accuracy is expressed as a mean (absolute value) percentage difference between mean quantitative value of specific controls and the target value. Precision is expressed as the coefficient of variation (CV) of that value.

13. Reporting of results

Urine samples with GHB present at a concentration above 10 µg/mL should be reported as positive for GHB. Blood samples with GHB present at a concentration above 5 µg/mL should be considered as positive for GHB and a quantitative value should be reported. The numbers are due to the fact that GHB is an endogenous compound.

14. References

"A solid phase method for gamma-hydroxybutyrate (GHB) in blood, urine, vitreous or tissue without conversion to gamma-butrylactone (GBL)", Developed by Joseph A. Crifasi, Saint Louis University health science center; Published on UCT website. https://www.unitedchem.com/sites/default/files/GHB-

GBL_in_Blood_2.pdf

"A solid phase extraction method for the determination of gamma-hydroxybutyrate (GHB) in urine without conversion to gamma-butrylactone (GBL)", Developed by Chester J. Kitchen, Thomas F. August and Michael Telepchak. US patent number 6,156,431.

Rev. #	History
2	Formatting and verbiage changes throughout. Section 7 – replaced methanol with water. Changed ethylene glycol internal standard to GHB-D6. Added multipoint calibration and external controls (Utak or in-house controls). SPE columns were added to procedure. Changed title of SOP to 'Gamma-Hydroxybutyric Acid (GHB) Analysis.'