1.0 PRINCIPLE

Gamma-hydroxy butyrate (GHB) is a central nervous system depressant agent acting as a GABA-receptor agonist. The compound can be used as a psychotropic agent, and has been utilized in DFSA cases. GHB is an endogenous compound produced from succinyl semialdehyde by the action of an NADPH-dependent also-keto reductase, and may be present in forensic samples, generally at levels well below 1 mg/L (Note: Postmortem samples may have considerably higher levels, as a consequence of oxidation of endogenous GABA). When present in forensic samples as a function of exogenous ingestion, GHB levels may range well above 10 mg/L.

GHB may be demonstrated in a screen assay as a function of extraction from a mildly acidic buffer dilution of sample, into an organic phase, with subsequent derivitization with BSTFA, forming the bis-TMS derivative. Qualitative identification of GHB is accomplished by comparison of retention time and ion ratios against a single-point calibrator (10 mg/L). Analytical validity of the batch is demonstrated by multiple-point matrix-specific controls ranging from 2 to 40 mg/L.

2.0 SPECIMEN

Specimens requiring screening for GHB are listed by lab case number on the clip board marked "GHB Screen List" which is maintained in the Toxicology Instrument room . Analysts preparing a batch for analysis should generate their batch sample list (see form 22.1 "GHB Screen Batch Worklist, appended to this document).

2.1 All evidence transfers, either between individuals or between an individual and a storage location must be documented on the Chain of Custody for the case, either in the LIMS, or on hard-copy COC document maintained in the Case Jacket.

2.2 When not in the sampling or aliquot process, samples in the Toxicology section must be stored in the secure and locked Toxicology evidence storage room.

2.3 Samples must be maintained in such a manner so that they are protected from contamination or deleterious change. Depending on the nature of the sample, this may mean refrigeration or freezing when not in the analytical process.

2.4 When aliquotting of samples is completed, the samples must be maintained "Under Proper Seal." This is interpreted to mean that the sample, or a container in which the sample is kept, is sealed with tamper-evident tape, with the initials and date of

person placing the seal clearly marked on, or proximate to that seal.

2.5 Samples are maintained in the Toxicology Section for 8 weeks after case is completed, in the absence of notification of any legal action, or other reason to maintain the samples. After this period, samples are discarded in the appropriate medical waste disposal container. Sample from fatalities, or cases with requests for retention are maintained by the laboratory. Sexual assault cases, or Drug-facilitate Sexual assault cases are returned to the submitting agency.

3.0 EQUIPMENT:

GC/MS and associated data station/computer (HP6890/5973 or equivalent)
General laboratory glassware and equipment
Micro-vial centrifuge

4.0 REAGENTS:

4.1 Reagents available as stock items:

Ethyl acetate (HPLC grade or equivalent)
BSTFA with 1% TMCS
GHB Stock: 1.0 mg/mL
Internal Standard Stock; Ethylene glycol; 1 mg/mL
Note: either propylene glycol or glycerol may be used as an alternate internal standard on a *per batch* basis.
Sodium Acetate (Baker reagent grade or equivalent)

4.2 Reagents prepared in the Toxicology Laboratory:

4.2.1 0.2 M NaOAc Buffer; pH 4.6/I.S. solution
(1.6 g NaOAc/90 mL H_2O, pH to 4.6; add 5.0 mL I.S. solution (1.0 mg/mL I.S.), q.s. to 100 ml with H_2O)

Note: Reagent Preparation and Validation is documented in the Toxicology "Reagent Preparation/Validation Logbook" maintained in the Toxicology section. Validation is addressed in section 4.5, below.

4.3 Calibrators and Internal Standard:

Note: Preparation of all calibrator and control solutions is documented in the "Calibrator and Control Preparation Log" (maintained in the Toxicology Wet Laboratory)

Ethylene Glycol internal standard; 1 mg/mL
(Baker Reagent Grade or equivalent) 0.1g/100mL H_2O
Propylene Glycol internal standard; 1 mg/mL
(Baker Reagent Grade or equivalent) 0.1g/100mL H_2O
Glycerol internal standard; 1 mg/mL
(Baker Reagent Grade or equivalent) 0.1g/100mL H_2O
GHB Calibrator stock solution 1 mg/mL
(Baker Reagent Grade or equivalent) 0.1g/100mL H_2O

4.4 Controls:

GHB Control stock solution 1 mg/mL
(Baker Reagent Grade or equivalent) 0.1g/100mL H_2O

Each batch incorporates 4 or more controls prepared (spiked) in blank blood and/or urine (as appropriate to batch makeup) prior to extraction as detailed in "Procedure," (section 5.0) below:

Blood and Urine: GHB controls: 2 mg/L (optional)
5 mg/L
10 mg/L
20 mg/L
40 mg/L(optional)

Acceptable control performance in this screen procedure is detailed in section 7.4, below.

4.5 Validation of Reagents

Validated reagents are marked with a green dot, detailing the specific procedure for which the reagent was validated, and the batch on which that process was documented. Newly prepared reagents may be evaluated for validity on an analytical batch, prior to any consideration of sample results. Acceptable performance of all batch control materials and overall batch acceptability (although individual samples may fail) is considered as validation of reagents. Reagents so validated are marked with a green sticker as noted above. Preparation of reagents, and their validation is documented in

the Toxicology Section Reagent Preparation Validation Logbook, maintained in the Toxicology laboratory.

5.0 PROCEDURE

Note: Departure from procedures as specified in this SOP is not anticipated. Should an issue arise that may require such a departure, the issue must be raised with the Section Supervisor, Quality Manager and/or the Director. If the proposed change will not present a change of such a magnitude so as to require validation, the change may be approved, and the Director will modify and re-issue the SOP accordingly.

Any such procedural changes would be subject to the review process afforded by the quality control measures of the analytical scheme described herein. Hence, any modification or change that produces an unexpected deleterious effect on the analytical procedure would be expected to trigger analysis or batch failure in the QC review stages.

5.1 Batch Format: Screen batches for GHB should follow the format indicated below: Note that samples are analyzed in duplicate.

Matrix blank
Calibrator; 10 mg/L
Matrix blank
Control 1
Control 2
Blank
Sample 1 rep 1
Sample 1 rep 2
Samples 2-10 (or fewer) Rep 1 and 2
Control 3
Control 4 (Final control, in the absence of additional samples)
Blank
Additional Samples (10 or fewer)
Additional (Final) Control

Note: Three of the four controls must be 5, 10, and 20 mg/L. The fourth may be either 2 or 40 mg/L. Additional controls may be added as appropriate per the discretion of the analyst.

5.2 Add 0.2 mL blank urine or blood to each properly labeled blank, calibrator and control 1.5 mL microcentrifuge vial, using an automatic dispensing pipette.

5.3 Add 0.2 mL sample to each properly labeled 1.5 mL microcentrifuge vial, using an automatic dispensing pipette.

5.4 For each Sample, Control or Calibrator; add 0.2 mL 0.2M NaOAc pH 4.6 buffer/Internal Standard solution to a labeled microcentrifuge vial, using an automatic dispensing pipette.

5.5 For each Calibrator (10 mg/L GHB), add 20 uL GHB/H_2O "Working Stock" solution (0.1mg/mL GHB/H2O) using a 50 ul analytical syringe.

5.6 For each Control; add GHB/H2O "Working Stock" solution (0.1 mg/mL GHB/H2O) using an appropriate analytical syringe as follows:

Conc. GHB	Amount of GHB Stock
2 mg/L	4 uL
5 mg/L	10 uL
10 mg/L	20 uL
20 mg/L	40 uL
40 mg/L	80 uL

5.7 Add 0.4 mL 10% EtOAc to each tube, using an automatic dispensing pipette. Cap and vortex each tube for ~ 20 sec.

5.8 Centrifuge all tubes using a bench-top microcentrifuge for ~1 min.

5.9. Carefully withdraw a 100 uL aliquot of the upper, organic layer from each tube, using an automatic pipette, and transfer to an appropriately labeled glass tube.

5.10 Evaporate the organic phase just to dryness using a stream of dry nitrogen (Turbo-vap apparatus, or equivalent)

5.11 Add 20 uL BSTFA/TMCS and 150 uL EtOAc to each tube, using automatic pipettes, mix briefly.

5.12 Cap and heat all samples, calibrators and controls for 10 minutes at 70^{o} C in a dry-block incubator. Reserve the vials for GCMS analysis.

6.0 CHROMATOGRAPHY AND MASS SPECTROSCOPY

6.1 Instrument and Setup: (see Appendix 1)

GCMS/Autosampler: (Hewlett-Packard 6890/5973, or equivalent)
Column: 30M RTX-5MS (0.25 mm ID; 0.25 micron film)
Inj. Temp 250^{o}
Oven: 60^{o}, 2.0 min; 25^{o}/min to 250^{o}
10.6 min total run time
1 ul inj volume
Note: the above parameters are guides minor changes to these parameters are acceptable if demonstracted though acceptable results of normal calibrator and controls.

6.2 Injection sequence**;** Samples are injected on the GC/MS in the following sequence: (see appendix 2 for an example of a GHB Screen batch Worklist)

Calibrator
Matrix blank
Control High
Control Low
Blank
Sample 1 rep 1
Sample 1 rep 2
Samples 2-10 Rep 1 and 2
Blank
Control High
Additional Samples
Final Control

6.3 DETECTION AND IDENTIFICATION

Determination of the presence of GHB in the sample extract is identified by appearance and ratio of the 3 ions characteristic of GHB at the appropriate retention time, hence both retention time (a GC characteristic) and fragmentation pattern and ratio (MS characteristics) are used as the basis of qualitative identification. For a positive Identification of GHB, the retention time must be within 5% of the corresponding analyte in the calibrator injection, and the ion ratios for both qualitative ions must be within 20% of the corresponding ratio calibrator sample.

6.4 CALIBRATION AND QUANTITATION

6.4.1 Calibration

Calibration for each batch is done independently. Hence, no sample analysis conducted under Toxicology/CS Lab guidelines an historical calibration curve. Calibration is accomplished by the incorporation into the sample procedures of a blank sample of the matrix being analyzed that has a known quantity (10 mg/L GHB) in addition to the internal standard. The response of the system to this calibrator, and the assumption of a 0 response to a 0 concentration, defines a run-specific standard curve that is used as the basis for the quantitative calculation in all controls and samples. The system is therefore “single-point calibration, multi-point control”.

7.0 QUALITY CONTROL AND RUN EVALUATION

7.1 Verification of Vial Sequence: The vial sequence is checked both prior to and after the injection of samples when the auto injector is used. The check after the injection of samples is documented on the run summary sheet.

7.2 Chromatography - Evaluation and Acceptance Criteria: Chromatographic quality is evaluated for each peak. While general guidelines are that a peak should be symmetrical, and be resolved to baseline on at least one side, with 90%

resolution on the other side, significant departures from those guidelines may be experienced with forensic samples. In many cases, chromatographic quality will warrant rejection of the chromatographic run, or specific samples, by the operator. Any such action should be clearly documented on the batch summary sheet. Questionable chromatographic peak shape, resolution, or other problems with chromatography can be discussed with the Director, Section Supervisor or Quality Manager.

7.3 Evaluation of Potential Carryover

Carryover in the chromatographic quality is evaluated by injection of a blank sample immediately following the calibrator. Carryover of greater than 2% (40 ng/mL) requires batch rejection, and remedial action for the instrument (e.g. replacement of injector insert, new septum and perhaps column trim or even replacement). Demonstrated carryover of less than 2% will require operator consideration with regard to the potential for effects on specific samples, and may require re-extraction of specific samples. Carryover is further evaluated on a per sample basis by the requirement that quantitative results between replicates agree within 20%. Any significant carrover effect should cause the first of the two replicate samples to exceed the second by an amount in excess

of the 20% differential. If not, any carryover may be considered inconsequential. In practice, when a question of potential carryover exists, the potentially affected sample replicates may be repeated at the end of the batch.

7.4 Control Results: For a GHB Screen batch to be considered acceptable, control results must be as follows:

Control	Acceptable Performance
2 mg/L	Negative or GHB present; < 10 mg/L
5 mg/L	GHB present; < 10 mg/L
10 mg/L	GHB present
20 mg/L	GHB present; > 10 mg/L
40 mg/L	GHB present; > 10 mg/L

7.5 Internal Standard: Minimal acceptable internal standard abundance is 1E5for the internal standard. For any individual injection to be acceptable, the internal standard abundance must be at least 20% of the corresponding abundance in the calibrator, and greater than the minimal abundance noted

above.

7.4 LINEARITY: Linearity of the calibration curve is demonstrable in each batch, for each analyte as a function of quantitative results of control materials.

7.5 SENSITIVITY- LIMITS OF DETECTION (LOD) and QUANTITATION (LOQ): For the purposes of this procedure, the LOD and LOQ are defined as equal to the lowest concentration of the lowest control. Qualitative Identification and/or Quantitative analysis below the concentration of the low control may be accepted on a case by case basis with the concurrence of the analyst, technical reviewer, Director and/or Quality Manager.

7.6 ACCURACY AND PRECISION:Accuracy is expressed as a mean (absolute value) percentage difference between mean quantitative value of 10 reps of the specific control, and the target value. Precision is expressed as the CV of that value

7.7 SPECIFICITY: Specificity is a function of both the resolution of target analyte during the analytical process, and the mass spectral fragmentation that analyte molecules undergo during the instrumental analysis. There has been no report of any material other than GHB that elutes within 5% of the retention time of known standard materials, and produce the same fragmentation ions and ratios.

7.8 REPORTING OF RESULTS: GHB screen analysis batches, containing controls and calibrators data and summary documentation are maintained in the Toxicology Laboratory. Specific chromatograms, and a copy of the batch summary page for each case are filed in the appropriate case file. Samples with GHB present at a concentration above the cut-off (10 mg/L) should be reserved for confirmatory, quantitative analysis. Samples for which GHB was either absent, or present at a concentration less than 10 mg/L may be reported as: “GCMS Screening for GHB was Negative.” (Note; such sample may, at the discretion of the analyst, be reserved for confirmatory, quantitative analysis.)

Procedural Uncertainty is reported with all quantitative results, and is calculated and tabulated annually for each analytical method, (See SOP TX-19 section 6.3).

8.0 QUALITY ASSURANCE: Quality Assurance is provided by the multiple layers of

checks that are performed both during and after analysis. Specifically:

8.1 The GC/MS run is thoroughly checked by the operator, including vial position on the autosampler, both prior to and following the injection of samples.

8.2 The GC/MS run is reviewed and signed off by a reviewer distinct from the operator, with this review including an evaluation of qualitative and quantitative (where applicable) results, including:

a. Control Results
b. Chromatographic Characteristics
c. Transcription errors

8.3 All results, as transcribed in the Case Summary Form are checked against the original run summary sheet during the process of report preparation, and during the administrative review of case results.

8.4 The original run is compared to the Final Report during the Final Director's review, prior to case sign-off.

9.0 SOURCES OF ERROR

The utilization of 3-ion SIM methodology, with reference to procedural, controls and calibrators yields qualitative drug identification with essentially no uncertainty. Screen results are reported only if negative. Presumptive positive results are subjected to quantitive confirmatory analysis.

10.0 REFERENCES

10.1 Villain, M., Cirimele, V., Ludes, B. and Kintz, P. Ultra-rapid procedure to test for gamma-hydroxybutyric acid in blood and uring by gas chrotography-mass spectrometry. J. Chromatogr B Analyt Technol. Biomed Life Sci. 2003 Jul 15; 792(1):83-87

10.2. Elian, A. A novel method for GHB detection in urine and its application in drug-facilitated secual assaults. Forensic Science International 2000; 109: 183-187

Appendix 1:

GC/MS temperature program specifications: note slight variations on this temperature program are acceptable as required by changes in the instrument conditions, such as a clipped column.

Temperature program	
Initial temp	60° C
Initial Time	2.00 min
Rate	25°/min
Final Temp	250°C
Final Hold	1 min
Post temp	0°C
Post time	0 min
Run Time	10.6 min
Inlet/Flow/Injection	
Mode	Split
Initial temp	250°c
Pressure	8.17 psi
Pulse pressure	25 psi
Pulse time	0.50 min
Split Ratio	2.02:1
Split Flow	2.0 m l/min
Total flow	5.5 mL/min
Gas type	Helium
Injection volume	1 microliter
Post injection solvent wash	A - 2, B - 2
Analysis	
Tune file	ATUNE
Acquisition mode	SIM
Solvent delay	4.5 min
M/Zs group 1	191,133, 103
Start time	4.5 min
M/Zs group 2	233, 233, 204, 117
Start time	6.0 min
Dwell	20

MS Quad	150°C
MS Source	230°C

GHB Batch Summary

Batch ID:

Sample (Case Number)	Autosampler vial Number	Specimen (Matrix)	Specimen Volume, (ml)	Cal. Stock Vol (ul)	Contr. Stock vol. (ul)	Int. Std. Violume (ul)	Analyte	Theoretical Concentration (ug/ml)	Observed Concentration (ug/ml)	Percent Recovery (Acceptable:80 - 120)	Notes
Conditioner	1	Blood	1	40		50	GBL				
Col 2							GBL				
Blank*	4	Blood	1	0		50	GBL	0.0	ND		
Col 2							GBL	0.0	ND		
5 Calibrator	5	Blood	1	5		50	GBL	5.00			
Col 2							GBL	5.00			
10 Calibrator	6	Blood	1	10		50	GBL	10.0	10.0		
Col 2							GBL	10.0	10.0		
20 Calibrator	7	Blood	1	20		50	GBL	20.0	20.1		
Col 2							GBL	20.0	20.1		
40 Calibrator	8	Blood	1	50		50	GBL	40.0	40.6	101.5	
Col 2							GBL	40.0	40.4	101.0	
80 Calibrator	9	Blood	1	80		50	GBL	80.0	80.5	100.6	
Col 2							GBL	80.0	80.2	100.3	
Negative control	10	Blood	1			50	GBL	0.00	ND	#VALUE!	
Col 2							GBL	0.00	ND	#VALUE!	

Control 15	11	Blood	1		15	50	GBL	15.0	14.2	94.7	
Col 2							GBL	15.0	13.4	89.3	
Control 15	12	Blood	1		15	50	GBL	15.0	14.2	94.7	
Col 2							GBL	15.0	12.2	81.3	
Control 10 (UTAK)	13	Blood	1		10	50	GBL	10.0	8.66	86.6	
Col 2							GBL	10.0	8.42	84.2	
Control 30	14	Blood	1		30	50	GBL	30.0	30.8	102.7	
Col 2							GBL	30.0	29.5	98.3	
Control 30	15	Blood	1		30	50	GBL	30.0	27.0	90.0	
Col 2							GBL	30.0	28.8	96.0	

Samples Extracted by: _____ Date: _____ GC Run Date: _____
Vial position verified prior to sample removal _____
Calibration correlation coefficient (Acceptable r > 0.98): Col 1_____ Col 2_____ 0.99 Col 2 0.99 *Carryover check: GBL < 5 ug/ml ? ____ Yes ___ No
** Acceptability; Peak Shape, Retention Time, IS Area, all must be acceptable.
ND: Not Detected Neg.: negative
Analyst Review by: _____ Date: _____ Run Accepted? ___ Yes ___ No Analyst Comments: _____ All cases are negative for GHB in this batch.
Technical Review by: _____ Date: _____ Run Accepted? ___ Yes ___ No Reviewer Comments:_____
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