Name of Procedure:

Toxicology
Extraction of Gamma Hydroxybutyric Acid (GHB) in Blood, Urine and Other Fluids

Suggested Uses:

This is an extraction procedure for Gamma Hydroxybutyric Acid (GHB) in blood, urine, and other fluids. This procedure is designed to extract Gamma Hydroxybutyric Acid for confirmation by gas chromatography/mass spectrometry. The extraction procedure targets Gamma Hydroxybutyric Acid at a concentration of 1.0 microgram/mL in a 1.0 mL blood sample, at 5.0 micrograms/mL in a 0.2 ml urine sample, or another fluid at an appropriate volume. Calibration standards must be utilized for quantitative analysis. Additional calibration standards at concentrations not listed may be used. 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.

Apparatus Needed to Perform Procedure:

Test tubes, 16 x 125, 13 x 100, 12 x 75
Test tube caps or stoppers
Vortexer
Test tube rocker
Centrifuge
Eppendorf pipettes
Pipet tips
Pasteur pipets
Volumetric flasks
Zymark TurboVap LV
Nitrogen

Reagents Needed:

Analytical Grade Drug Standard
    Gamma Hydroxybutyric Acid, Na (GHB)
Internal Standard
    Gamma Hydroxybutyric Acid-D_6 , Na (GHB-D_6)
Sulfuric acid
Deionized water

Methanol
Ethyl Acetate
Bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (BSTFA w/1% TMCS)

Sulfuric Acid 0.1N

a. To approximately 75 mL of deionized water add 280 microliters concentrated sulfuric acid.
b. Dilute to 100 mL with deionized water and mix.
c. Stability - indefinite.

Internal Standard Solution

Gamma Hydroxybutyric Acid-D_6 (GHB-D_6) 100 micrograms/mL: Purchase or prepare an internal standard solution that contains 100 micrograms of GHB-D_6/mL. Label the flask "100 micrograms/mL GHB-D_6 Internal Standard." Include on the label initials, the date prepared, and the expiration date obtained from the ampule.

Example: From an ampule that contains 1.0 mg/mL GHB-D_6, add 1.0 mL to a 10.0 mL volumetric flask, and dilute the flask to volume with methanol.

Calibration Solutions

a. GHB Stock Calibrator Solution: Prepare or purchase a GHB stock calibrator solution that contains 1.0 milligram of GHB/mL in methanol. Label this solution "1.0 mg/mL GHB Stock Calibrator" and include initials, the date prepared, and the expiration date which is determined from the expiration date of the acquired drug standard or one year from date of preparation if prepared from solid GHB-sodium.

   Example of preparation: Add 10 milligrams of GHB Sodium to a 10 ml volumetric flask and dilute to volume with methanol.

   A GHB stock verification solution must be prepared using the same procedure to prepare the stock calibrator. Label this solution appropriately.

b. GHB Working Calibrator Solutions: From the GHB Stock calibrator solution perform dilutions to obtain solutions that contain 100 micrograms/mL of GHB in methanol, 50 micrograms/mL of GHB in methanol, and 10 micrograms/mL of

GHB in methanol. Label these solutions "(concentration) GHB Working Calibrator" and include initials, the date prepared, and the expiration date which is determined from the expiration date of the stock calibrator solution. Purchased commercial standards of GHB may be used at any of these concentrations.

Examples: 100 micrograms of GHB/mL-Dilute 1.0 milliliter of the GHB stock solution to 10.0 milliliters with methanol. 50 micrograms of GHB/mL-Dilute 500 microliters of the GHB stock solution to 10.0 milliliters with methanol. 10 micrograms of GHB/mL-Dilute 100 microliters of the GHB stock solution to 10.0 milliliters with methanol.

Working verification solution(s) must be prepared using the same procedure to prepare the working calibrators. Label these solution(s) appropriately.

c. Blood Calibration/Verification Standards:
   1. 1.0 microgram GHB/mL: add 100 microliters of the 10 micrograms of GHB/mL working calibrator and 200 microliters of the GHB-D_6 internal standard to a clean test tube. Evaporate to dryness at 40°C or less. Add 1.0 mL of blood to the test tube and vortex.
   2. 5.0 micrograms GHB/mL: add 100 microliters of the 50 micrograms of GHB/mL working calibrator and 200 microliters of the GHB-D_6 internal standard to a clean test tube. Evaporate to dryness at 40°C or less. Add 1.0 mL of blood to the test tube and vortex.
   3. 10.0 micrograms GHB/mL: add 200 microliters of the 50 micrograms of GHB/mL working calibrator and 200 microliters of the GHB-D_6 internal standard to a clean test tube. Evaporate to dryness at 40°C or less. Add 1.0 mL of blood to the test tube and vortex.
   4. 25.0 micrograms GHB/mL: add 250 microliters of the 100 micrograms of GHB/mL working calibrator and 200 microliters of the GHB-D_6 internal standard to a clean test tube. Evaporate to dryness at 40°C or less. Add 1.0 mL of blood to the test tube and vortex.
   5. 50.0 micrograms GHB/mL: add 500 microliters of the 100 micrograms of GHB/mL working calibrator and 200 microliters of the GHB-D_6 internal standard to a clean test tube. Evaporate to dryness at 40°C or less. Add 1.0 mL of blood to the test tube and vortex.
   6. 100 micrograms GHB/mL: add 1.0 milliliter of the 100 micrograms of GHB/mL working calibrator and 200 microliters of the GHB-D_6 internal standard to a clean test tube. Evaporate to dryness at 40°C or less. Add 1.0 mL of blood to the test tube and vortex.

d. Urine Calibration/Verification Standards:
   1. 1.0 microgram GHB/mL: add 20 microliters of the 10 micrograms of GHB/mL working calibrator and 40 microliters of the GHB-D_6 internal standard to a clean test tube. Evaporate to dryness at 40°C or less. Add 0.2 mL of urine and 0.8 mL of deionized water to the test tube and vortex.
   2. 5.0 micrograms GHB/mL: add 100 microliters of the 10 micrograms of GHB/mL working calibrator and 40 microliters of the GHB-D_6 internal standard to a clean test tube. Evaporate to dryness at 40°C or less. Add 0.2 mL of urine and 0.8 mL of deionized water to the test tube and vortex.
   3. 10 micrograms GHB/mL: add 200 microliters of the 10 micrograms of GHB/mL working calibrator and 40 microliters of the GHB-D_6 internal standard to a clean test tube. Evaporate to dryness at 40°C or less. Add 0.2 mL of urine and 0.8 mL of deionized water to the test tube and vortex.
   4. 20 micrograms GHB/mL: add 400 microliters of the 10 micrograms of GHB/mL working calibrator and 40 microliters of the GHB-D_6 internal standard to a clean test tube. Evaporate to dryness at 40°C or less. Add 0.2 mL of urine and 0.8 mL of deionized water to the test tube and vortex.
   5. 50 micrograms GHB/mL: add 200 microliters of the 50 micrograms of GHB/mL working calibrator and 40 microliters of the GHB-D_6 internal standard to a clean test tube. Evaporate to dryness at 40°C or less. Add 0.2 mL of urine and 0.8 mL of deionized water to the test tube and vortex.
   6. 100 micrograms GHB/mL: add 400 microliters of the 50 micrograms of GHB/mL working calibrator and 40 microliters of the GHB-D_6 internal standard to a clean test tube. Evaporate to dryness at 40°C or less. Add 0.2 mL of urine and 0.8 mL of deionized water to the test tube and vortex.
   7. 200 micrograms GHB/mL: add 400 microliters of the 100 micrograms of GHB/mL working calibrator and 40 microliters of the GHB-D_6 internal standard to a clean test tube. Evaporate to dryness at 40°C or less. Add 0.2 mL of urine and 0.8 mL of deionized water to the test tube and vortex.

Procedure:

1. Sample Preparation:
   Blood - To a clean test tube add 200 microliters of the 100 micrograms GHB-D_6/mL internal standard solution. Evaporate to dryness at 40°C or less. Add 1.0 mL of blood to the test tube and vortex.
   Urine - To a clean test tube add 40 microliters of the GHB-D_6/mL internal standard solution. Evaporate to dryness at 40°C or less. Add 0.2 mL of urine or other liquid and 0.8 mL of deionized water to the test tube and

vortex.

2. Extraction Proceedure:
   a. Add 250 microliters of cold 0.1N H_2SO_4 to the urine or blood sample.
   b. Mix/vortex.
   c. Add 4 milliliters of ethyl acetate and cap the test tube securely.
   d. Place the test tube(s) on the test tube rocker and allow to mix for 10 minutes. After removing the test tube(s) from the rocker centrifuge the test tubes for 5 minutes.
   e. Transfer the organic layer (upper) into a clean test tube.
   f. To the test tube containing the blood or urine, add 4 milliliters of ethyl acetate and cap the test tube securely.
   g. Repeat steps d and e.
   h. Transfer the organic layer into the test tube that contains the previously transferred organic layer from step e.
   i. Evaporate the organic layers to dryness at 40°C or less. This test tube will contain the extracted material.

Post Extraction Procedure:

1. Add 50 microliters of BSTFA w/1% TMCS to each test tube and cap securely.

2. Heat for 15 minutes at 80°C.

3. Remove from heat and allow the mixture to cool before analysis.

4. Examine the collected fraction utilizing the ghbblood, ghburine, ghbfs or other appropriate methods on the gas chromatograph/mass spectrometer.

Quality Control:

Quality control is verified for each extraction by utilizing the appropriate internal standard. For each set of extractions an appropriate blank must be extracted as a negative control. Reported quantitative values must be based on a calibration curve using at least four calibration standards, and at least one verification standard must be extracted to verify the calibration curve. The verification standard must quantify within +/- 20% of the target for quantitative values to be reported.

Personnel Safety Concerns:

1. When working with biohazardous samples use protective measures, such as gloves, eye protection, and work with the samples in a biosafety hood.

2. Sulfuric acid, ethyl acetate, and methanol should be handled in a fume hood with eye protection.

Literature References:

Fiona J. Couper 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, pp. 1-7.

Rachel R. McCusker, Helen Paget-Wilkes, Chris W. Chronister, Bruce A. Goldberger, and Mahmoud A. ElSohly; "Analysis of Gamma-Hydroxybutyrate (GHB) in Urine by Gas Chromatography-Mass Spectrometry," **Journal of Analytical Toxicology**, Vol. 23, September 1999, pp. 301-305.

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