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Abstract
An effective method for the determination of gamma-hydroxybutyric acid (GHB) in human plasma is described that utilizes a simple liquid-liquid extraction procedure and gas chromatography–positive ion chemical ionization-mass spectrometry (GC-PCI-MS). The method has been used to study the stability of plasma GHB under several storage conditions. Following the extraction with acetonitrile, GHB and deuterated GHB (GHB-d₆) were derivatized with N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA). After the separation on a capillary GC column, the derivatives were ionized with ammonia reagent gas and analyzed by MS. The lower limit of quantitation in 100 μL of plasma was 2.5 μg/mL, over a range from 2.5 to 250 μg/mL. The coefficients of variation did not exceed 3.9% and the mean measured concentrations did not deviate more than 8% from the target for both intra- and interassay precision and accuracy. Plasma GHB was found to be stable at -20℃ for up to 9 months, at room temperature for 48 h, and after 3 freeze/thaw cycles. It was also found to be stable in processed samples stored at room temperature for 5 days and for 15 days at -20℃.

Introduction
Gamma-hydroxybutyric acid (GHB) is a central nervous system depressant and well-known for its sedative and anesthetic properties (1,2). GHB had been used for the treatment of alcohol withdrawal, narcolepsy and induction of anesthesia (3). GHB can cause nausea, drowsiness, dizziness, bradycardia, seizures, respiratory depression, and coma (4). Because GHB and its precursor gamma-butyrolactone and 1,4-butanediol are easily acquired, abuse of GHB has increased dramatically (4–6). The ability of GHB to increase the secretion of growth hormone leads to its misuse by body-builders as an alternative to steroids (7). Because of its euphoric and sedative properties, GHB is sometimes associated with sexual assaults ("date rape"). In a study of 1179 urine samples from alleged victims of sexual assault, GHB was detected in 48 samples (8). The growing problems associated with GHB present a challenge to those in the toxicology and clinical fields.

To meet the need for an efficient method for the determination of GHB, many analytical methods have been reported. Most methods involve extraction by liquid–liquid or solid-phase procedures and analysis of GHB by high-performance liquid chromatography, gas chromatography (GC), or GC–mass spectrometry (GC–MS) (9–18).

The procedure presented here provides a simple and reliable method for direct quantitation of GHB in plasma at concentrations above the normal endogenous levels. GHB-d₆ was used as an internal standard. A 100-μL volume of plasma was extracted with acetonitrile. N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) was used for the derivatization. The trimethylsilyl derivatives of GHB and GHB-d₆ were separated by a GC capillary column, then ionized by ammonia positive ion chemical ionization, and detected by GC–MS.

Materials and Methods
Chemicals and reagents
GHB (1.0 mg/mL in methanol) and GHB-d₆ (1.0 mg/mL in methanol) were purchased from Cerilliant (Austin, TX). BSTFA with 1% trimethylchlorosilane (TMCS) was purchased from Pierce Chemical Co. (Rockford, IL). All solvents (acetonitrile, methanol and ethyl acetate) were HPLC-grade and purchased from Burdick & Jackson (Muskegon, MI) or Fisher Scientific (Fairlawn, NJ).

Standards and controls
The GHB methanolic standard was appropriately diluted with methanol to prepare separate stock and working solutions

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that were then stored at −20°C. Calibrator samples were prepared by fortifying 100 μL of human plasma samples with GHB working solutions at 6 concentrations (2.5, 5.0, 10, 50, 100 and 250 μg/mL). The quality control samples were prepared using separate stock and working solutions to fortify 100 μL samples of human plasma with GHB at 4 concentrations (2.5, 5.0, 75 and 150 μg/mL). The plasma used for preparation of calibrators and controls is tested to demonstrate it is free of drugs routinely analyzed in our laboratory. The inability to totally exclude any detectable GHB is discussed more thoroughly in the Results and Discussion.

Sample preparation

One hundred microliters of plasma was transferred to a silanized glass tube. Fifty microliters of GHB-d6 solution (10 ng/μL) was added to each calibrator, quality control, and study sample. Samples were vortex mixed. Two milliliters of acetonitrile was added to each tube. The samples were mixed well and centrifuged at 2500 rpm for 5 min. Fifty microliters of the supernatant was transferred to a clean tube and dried under a stream of air at 40°C in a Zymark Turbo Vap® LV Evaporator (Hopkinton, MA). Tubes were removed and 100 μL of BSTFA was added. Tubes were tightly capped with Teflon screw caps and heated at 70°C for 15 min on a Dry Bath Incubator (Fisher Scientific, Fairlawn, NJ). After tubes were removed from the incubator and allowed to cool to room temperature, the extracts were transferred to autosampler vials for analysis by GC–MS.

Instrumentation and GC–MS analysis

The GC–MS system consisted of an HP 6890 GC system (Palo Alto, CA), an HP 7683 injector and autosampler, and an HP 5973 mass selective detector (MSD) equipped with an HP Xa6/400 computer with a Windows NT operating system and HP ChemStation operating software. A DB-5, 30-m × 0.32-mm i.d., 1-μm film thickness capillary column purchased from J&W Scientific Inc. (Folsom, CA) was employed. The MS was operated in the PCI mode with ammonia as the reagent gas. One microliter of extract was injected in the splitless mode and carried by ultra-pure helium gas at a constant flow rate of 2.5 mL/min. Inlet and transfer line temperatures were set at 250°C and 280°C, respectively. The column temperature ramp was 70°C for 0.5 min; increased to 100°C at 15°C/min, to 140°C at 25°C/min, and to 310°C at 35°C/min. The ion source temperature was set at 200°C. The run time of each injection was 9 min.

The protonated molecule ions at m/z 249 and 255 for GHB and GHB-d6, respectively, were analyzed by selected ion monitoring. The concentration of GHB was determined from the ratio of the peak area of GHB to the peak area of GHB-d6, and comparison of this ratio with the calibration curve that was generated from the analysis of human plasma fortified with known concentrations of GHB. The response curves (area response ratio vs. concentration of analyte) were weighted concentration-inversely (1/a) with a quadratic regression curve fit.

Results and Discussion

The mass spectrum resulting from ammonia positive ion chemical ionization of the trimethylsilylated GHB-d6 consists of a single abundant ion peak corresponding to the MH+ at m/z 249. Because the positive ion chemical ionization does not cause extensive fragmentation, a qualifier ion is not utilized.

To determine specificity, six blank plasmas from different human sources were analyzed. In each source of blank plasma a peak with the same ion current profile as GHB-d6 was found (Figure 1). When the area ratios of these peaks to the internal standard were compared to the area ratios of samples fortified with GHB at 2.5 μg/mL [the lower limit of quantitation (LLOQ)], they ranged in size from 18 to 26% of the LLOQ. The signal-to-noise appeared sufficient to allow the use of 2.5 μg/mL as the LLOQ, which had excellent signal and chromatographic peak shape (Figure 1). A few studies have reported use of LLOQs at 1 μg/mL (10,17). The peaks identified in blank samples in this study precluded use of a LLOQ that low. Previous studies (19,20) have suggested that endogenous GHB can be found at concentrations less than 0.02 nmol/mL (i.e., = 2 ng/mL), while the peaks identified in this study would approximate 600 ng/mL. Our current source of blank plasma is
out-dated blood bank plasma. Although this material is generally kept frozen from the time of collection, handling procedures that differ from those in the previous studies may account for the differences in apparent endogenous concentrations of GHB.

When recovery was evaluated at concentrations of 5 and 150 μg/mL, the same percentage recovery (43.5%) was obtained for both concentrations. Despite this low percent recovery, the data presented here demonstrated that the extraction efficiency of this method was reproducible and satisfactory.

The use of 2.5 μg/mL as the LLOQ was based on our specificity study, and the use of 250 μg/mL as the upper limit of quantitation (ULQ) was based on reported data. Couper (21) reported blood GHB ranged from 26 to 127 μg/mL from 8 drivers arrested for driving under the influence (DUI). Pearson (22) reported blood GHB ranged from 81 to 360 μg/mL from 11 DUIs. In our method validation, no carry-over was detected following the injection of ULQ. Though the ULQ could be extended to a higher concentration, it is more practical to set the ULQ at this level.

Because the detection limit of this method is at the concentration level of microgram per milliliter, ion current saturation is of greater concern. To avoid that happening, only 50 μL of the acetonitrile supernatant (2.5% of total volume) was transferred for drying and derivatization during sample preparation. This technique significantly reduced the amount of analytes injected onto the column and subsequently onto the detector.

When intra- and interassay precision and accuracy were evaluated at the concentrations of 2.5 (LLOQ), 5, 75, and 150 μg/mL, the coefficients of variation did not exceed 3.8%, and the mean concentrations did not deviate more than 8% from the target (Table I). The correlation coefficient of the calibration curve was 1.0000 applying the quadratic regression curve fit, which provided better correlation of the response to the concentration in this method.

The internal standard concentration in this method is 5 μg/mL. Although it is not in the midrange of the calibration curve, it does not affect the valuation of this method. The use of this concentration was mainly based on the economic standpoint. As the GHB-d₆ was supplied in 100 μg per ampule at the time of this method validation, more samples could be run with this lower concentration.

Stability of GHB in human plasma was studied under different storage conditions. As a subject of forensic investigation, GHB samples may need to be stored for a long period of time. The same sample may be used for multiple analysis, which means the sample may be repeatedly going through freezing and thawing cycles. Currently there are limited published data of long-term stability on plasma GHB under frozen condition. LeBeau et al. (23) reported that GHB was elevated in citrate-buffered whole blood stored at −20°C for a period of 6–36 months. They suggested the elevation was caused by the citrate-buffer.

Applying the method described here, the stability of plasma GHB was evaluated after storage at −20°C for nine months. Both concentrations of plasma GHB tested showed no significant change (Figure 2). It indicates that GHB is very stable in frozen condition. When evaluating the stability of GHB in human plasma after three freeze/thaw cycles, deviations of mean concentrations from the targets did not exceed 10% and deviations of mean concentrations from 0-h controls did not exceed 5%. Bench-top stability of GHB in human plasma was determined at room temperature for up to 48 h. Deviations of mean concentrations did not exceed 11% of the target and did not exceed 4% of 0-h controls.

In the laboratory, analysis sometimes is interrupted by instrument problems. In this situation, the analysis of processed samples will be delayed. To test stability under these conditions, processed samples were re-injected with fresh calibrators and QC samples after storing on the autosampler at room temperature for 5 days and then at −20°C for 15 days. Mean concentrations did not deviate more than 12.7% from the target.

Table I. Precision and Accuracy for Analysis of Plasma GHB

<table>
<thead>
<tr>
<th>Target Concentration (μg/mL)</th>
<th>Intra-assay</th>
<th>Interassay</th>
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<tbody>
<tr>
<td></td>
<td>% Target</td>
<td>% CV</td>
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<tr>
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<tr>
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<tr>
<td>150</td>
<td>99</td>
<td>2.4</td>
</tr>
</tbody>
</table>

* %Target and %CV were calculated based on the mean and standard deviation of 5 replicates.
* %Target and %CV were calculated based on the mean and standard deviation of 15 replicates from 5 different runs.

Conclusions

This study demonstrates that the GC-PCI-MS method can accurately and precisely quantitate GHB in human plasma at concentrations ranging from 2.5 to 250 μg/mL. The study also
demonstrates that the GHB in human plasma is stable under the following conditions: (1) storage at room temperature for up to 48 h; (2) after 3 freeze/thaw cycles; and (3) storage at -20°C for up to 9 months. Processed samples were shown to be stable when stored on an autosampler at room temperature for up to 5 days, or in a -20°C freezer for up to 15 days.

Acknowledgment

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References