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TECHNICAL NOTE

TOXICOLOGY

Phillip A. Stout,[1] *M.S.; Kelsie D. Simons,*[1] *M.S.; and Sarah Kerrigan,*[1] *Ph.D.*

Quantitative Analysis of Gamma-Hydroxybutyrate at Endogenous Concentrations in Hair using Liquid Chromatography Tandem Mass Spectrometry

ABSTRACT: A method capable of quantifying endogenous concentrations of gamma-hydroxybutyrate (GHB) in human head hair was developed and validated using liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS). Hair was digested under alkaline conditions, and GHB was isolated using liquid–liquid extraction. LC/MS/MS was performed using atmospheric pressure chemical ionization in the negative mode, multiple reaction monitoring, and deuterated internal standard (GHB-D_6). Linearity was observed between 0.1 and 100 ng/mg GHB (R^2 = 1.000). The limits of detection and quantitation in human hair were 0.2 and 0.4 ng/mg, respectively. Accuracy at 2 ng/mg and 10 ng/mg was determined to be 97% and 94%, and intra-assay CVs at these concentrations were 5.2% and 7.4% (n = 4). Beta-hydroxybutyrate (BHB), alpha-hydroxybutyrate, gamma-butyrolactone, and 1,4-butanediol did not produce an interference, and there was negligible ion suppression or enhancement from the matrix.

KEYWORDS: forensic science, toxicology, gamma-hydroxybutyrate, liquid chromatography/mass spectrometry/mass spectrometry, hair

The purpose of this study was to develop a quantitative procedure capable of detecting very low concentrations of gamma-hydroxybutyrate (GHB) that are present in the hair of drug-free individuals. GHB is both an endogenous neuromodulator and a central nervous system depressant capable of producing heavy sedation at recreational dosages. Although the prevalence of GHB in drug facilitated sexual assault (DFSA) cases is controversial, it is indisputably a drug of interest in this type of casework. Because of the relatively short half-life of the drug in blood and absence of useful metabolites, detection times in blood and urine are typically only 6–12 h (1,2). Delays in specimen collection following an allegation of sexual assault are not uncommon, and biological samples are not always collected within the necessary timeframe. Hair has been proposed as an alternative matrix because of the prolonged window of detection for most drugs. However, published data on endogenous GHB concentrations in hair are limited compared with other matrices. This is in part because of the relatively small number of published procedures with detection limits sufficiently low enough to detect GHB in hair at endogenous concentrations.

GHB is a schedule I central nervous system depressant drug that can produce effects ranging from euphoria, sedation, relaxation, bradycardia, and respiratory depression at low to moderate doses, to coma or death at high doses (3). In addition, GHB is clinically available as a schedule III drug (Xyrem®) for the treatment of cataplexy, a condition associated with 70% of narcoleptic patients (4). Recent studies have also indicated that GHB may have some promise in the treatment of Alzheimer's disease (5). More commonly, however, GHB has been used recreationally by young people, body builders, and as a weight loss supplement (4). In addition to its sedative effects, GHB is colorless and odorless, making it difficult to detect, and consequently, a drug of interest in DFSA cases.

Although GHB is a drug in its own right, it is also a metabolite of gamma-aminobutyric acid (GABA). As a consequence, it is detected in small quantities in a wide variety of biological samples (6). Because GHB is an endogenous substance that is also encountered in forensic casework following exogenous administration, differentiation of endogenous and exogenous concentrations in biological samples is important. To this end, many forensic laboratories will establish a reporting "cutoff" for GHB, above which the presence of the drug might suggest exogenous administration. Although endogenous or background concentrations of GHB in blood and urine have been investigated and reported, limited data are available for hair.

An investigation of endogenous GHB concentrations in blood was conducted by Shima et al. (7). Blood collected from 24 healthy volunteers indicated concentrations from 0.005 to 0.010 mg/L, with no obvious difference in concentration based on sex. Although there are a number of articles pertaining to endogenous GHB in blood, previous studies have mainly focused on stability of the drug during storage and factors leading to *in vitro* production, which can further complicate interpretation of the results. In a study of endogenous urinary concentrations of GHB, Kaufmann observed baseline concentrations up to 2.7 mg/L (n = 20) (8). In their study of 50 women, Crookes et al. reported slightly lower urinary concentrations ranging from 0.1 to 1.5 mg/L with a noted decrease in these concentrations with age (9). Shima et al. noted urinary concentrations ranging from 0.16 to 2.14 mg/L

[1]Forensic Science Program, College of Criminal Justice, Sam Houston State University, Box 2525, 1003 Bowers Blvd, Huntsville, TX 77341.

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in healthy human subjects ($n = 30$) and slightly elevated concentrations of 0.17 to 3.03 mg/L in diabetic subjects ($n = 20$), with statistical variation between sex (10). While Moriya et al. (11) reported no statistical differences based on sex, they reported higher urinary concentrations in smokers ($n = 4$) when compared to nonsmokers/nondrinkers ($n = 12$) and drinkers ($n = 4$). In contrast, in a longitudinal study of eight subjects (five males, three females), LeBeau et al. (12) determined endogenous GHB concentrations to be statistically different ($p < 0.001$) between sexes. In another study of urinary GHB concentrations in 100 forensic toxicology casework samples stored for up to a year, Kerrigan reported endogenous concentration in the range 0–7 mg/L, with a mean of 1.8 mg/L (13).

Following administration of GHB, the drug can be cleared from blood in 6 h and urine in 10 to 12 h (1,2). This may yield inconclusive results for DFSA cases in which there exists a significant delay between the alleged assault and collection of the specimen. In contrast, hair is a relatively inert matrix capable of retaining drug over time, thus increasing the window of detection for many drugs. Assuming an average growth rate of approximately 1 cm/month, segmental analysis may allow an approximate timeframe of drug exposure to be determined. However, the interpretive value of GHB determination in hair is still under investigation. In a case study of a suspected death from GHB toxicity, Kalasinsky et al. reported a concentration of 47 ng/mg in a washed root bulb indicating the utility of hair in cases of rapid death (14). Kintz et al. investigated endogenous GHB concentrations in the shaft of the hair, noting concentrations ranging from 0.5 to 12.0 ng/mg, with no difference in concentration based on hair color or sex ($n = 24$) (15). Furthermore, in their study of 61 subjects, Goullé et al. noted endogenous GHB concentrations ranging from 0.32 ng/mg to 1.86 ng/mg, similarly observing no significant differences based on hair color (16).

Our institution received Institutional Review Board approval to determine endogenous concentrations of GHB in a significantly large human population (>100). To complete this study, it was necessary to develop an analytical technique capable of detecting extremely low GHB concentrations (tenths of a nanogram per milligram of hair) in a complex matrix like hair. This report summarizes the assay development and analytical performance of such a procedure.

Methods

Reagents

Formic acid was purchased from Sigma-Aldrich (St. Louis, MO). Sodium hydroxide and acetone were purchased from Fisher Scientific (Pittsburgh, PA). Methyl alcohol, sulfuric acid, and ethyl acetate were purchased from Mallinckrodt Chemicals (Hazelwood, MO). GHB, GHB-D_6, 1,4-butanediol, and gamma-butyrolactone were purchased from Cerilliant (Round Rock, TX). (R)-2-Hydroxybutyric acid (β-hydroxybutyric acid) was purchased from Fluka (St. Louis, MO), and 3-hydroxybutyric acid (α-hydroxybutyric acid) was purchased from Sigma-Aldrich.

Instrumentation

Analysis was performed using a Shimadzu HPLC system (Columbia, MD) coupled with an Applied Biosystems API 3200 tandem mass spectrometer (Foster City, CA) with a heated nebulizer. The column used was a Phenomenex Luna 5 μm C18 Column (100 × 2.00 mm) with a Phenomenx HPLC Analytical Guard column (4.0 × 2.0 mm, C18) (Torrance, CA). The mobile phase consisted of 0.1% aqueous formic acid and methanol (90:10) and was run isocratically at a flow rate of 0.2 mL/min. Samples were injected onto the column using a Shimadzu Sil-20A HT autosampler equipped with two LC-20AT pumps.

Instrumental Optimization

Compound optimization was performed by direct infusion of GHB and GHB-D_6 (5 mg/L in mobile phase) using both a turbo ion spray (electrospray ionization) probe and a heated nebulizer (atmospheric pressure chemical ionization [APCI]) probe in both positive and negative modes. Product ion selection criteria was set to choose the two most intense ions with a minimum mass to charge ratio of 50 amu in Q3 to increase the specificity of the product ions and to try to minimize background noise. Source-dependent parameters were optimized by injecting 0.5 mg/L standards directly from the autosampler to the detector, bypassing the column.

To determine optimum source and ionization conditions, GHB standard, liquid–liquid extracts, digested hair extracts with only internal standard (5 ng/mg), and digested hair extracts with internal standard and GHB (10 ng/mg) were evaluated. The stability of extracts in mobile phase and deionized water was also evaluated over a period of 2 days to ensure that there was not significant loss of analyte in the mobile phase.

Collection and Decontamination

Hair was cut at the scalp from volunteers at the posterior vertex in accordance with well-established guidelines (17,18). This region reportedly has the highest percentage of hair follicles in the growth phase and shows less variation in growth rate based on sex or age. Following sample collection, samples were placed on foil and tied at the root end for identification purposes. The foil was then folded on all sides to secure and prevent loss of specimen, placed in an envelope, and stored at room temperature. Hair was partitioned into 3-cm segments and placed in a round bottom glass tube. Sufficient acetone was added to completely submerge the sample, and tubes were vortex mixed for 20 sec and allowed to sit for 2 min. After 2 min, the acetone was decanted and the hair was allowed to air dry in the glass tubes overnight.

Assay Optimization

Conditions necessary for the digestion of hair and subsequent extraction using liquid–liquid extraction were investigated. The most critical step involved the acidification of the digest prior to extraction. Briefly, 25 mg of hair was finely cut and aliquoted into disposable screw cap glass tubes. Digestion of the hair matrix was achieved using 0.5 mL of 1 M sodium hydroxide at 95°C for 15 min. Hair was allowed to cool to room temperature for 5 min. Varying amounts of sulfuric acid (1 M) were added, followed by 3 mL of ethyl acetate. Samples were immediately vortex mixed for 20 sec, centrifuged for 5 min at 1500 × *g*, and the supernatant organic layer decanted, evaporated, and reconstituted in mobile phase. To standardize the extractions, ion transitions for GHB (102.9–57.0 and 102.9–85.1) and the internal standard (109.0–61.1 and 109.0–90.0) were combined. Response ratios were compared at both 5 and 20 ng/mg. Subsequent experiments were performed in which variations in temperature for the digestion, as well as the duration of the digestion were also conducted.

Assay Validation

The assay was validated using fully optimized conditions. Head hair (25 mg) was digested using 0.5 mL 1 M sodium hydroxide solution at 75°C for 40 min. After cooling to room temperature, 0.6 mL 1 M sulfuric acid was added, followed by 3 mL ethyl acetate. Samples were immediately vortex mixed for 20 sec and centrifuged at $1500 \times g$ for 5 min. The organic supernatant was transferred to a conical glass tube and evaporated to dryness under air at room temperature. Extracts were reconstituted in 20 μL of mobile phase (0.1% formic acid in methanol, 90:10), and 15 μL was injected onto the column.

Under optimized conditions, analysis was performed on an API 3200 tandem mass spectrometer equipped with a heated nebulizer (APCI) probe, in negative mode. The heated nebulizer was operated at a temperature of 550°C with curtain gas set to 50.0 psi, nebulizer current at −4.0 μA, ion source gas (gas 1) at 80.0 psi, and CAD gas at 5.0 psi. Q1 conditions were optimized for the deprotonated $(M–H)^-$ molecular ion for both compounds. Ions were examined in multiple reaction-monitoring (MRM) mode, observing the transitions from 102.9 to 57.0 and 85.1; and 109.0 to 61.1 and 90.0 for GHB and GHB-D_6, respectively. Quantitative analysis was performed by linear regression of using the ANALYST 1.4.2 software. (Applied Biosystems).

Methanolic working standards for GHB and GHB-D_6 were used to fortify 25 mg aliquots of hair. Calibrators were routinely prepared in the range 0.5, 1.0, 2.5, 5.0, 25.0 ng/mg, although concentrations as high as100.0 ng/mg and as low as 0.2 ng/mg were included for some evaluations. All samples were fortified with 20 μL of internal standard solution (0.01 mg/mL) to give a final GHB-D_6 concentration of 8 ng/mg. The hair samples selected for preparation of the calibrators contained no detectable GHB (less than the limit of detection [LOD]).

The LOD was determined empirically by fortifying 25 mg aliquots of hair with decreasing quantities of GHB. The LOD was defined as the lowest concentration of GHB that produced a signal to noise ratio of 3:1 or more at the expected retention time for the appropriate parent/daughter transition. The limit of quantitation (LOQ) was defined as the lowest concentration of GHB that produced a signal to noise ratio of at least 10:1 and calculated concentrations within 20% of the expected value. Accuracy was determined by fortifying hair (25 mg) with GHB at 2 ng/mg and 10 ng/mg. Precision was evaluated by replicate analysis ($n = 4$) of fortified hair samples at two independent concentrations.

Potential for interference by α-hydroxybutyric acid (AHB), β-hydroxybutyric acid (BHB), gamma-butyrolactone (GBL), and 1,4 butanediol (1,4 BD) was also evaluated. Automatic compound optimization was conducted using 10 μg/mL solutions of standard, with selection criteria for product ions being restricted to the two most intense ions with a mass to charge ratio of at least 50:1. Source-dependent parameters were subsequently optimized using flow injection analysis (FIA), and compounds were injected onto the column using the method created for each compound to observe retention times. All compounds were then injected at concentrations of 10 μg/mL with 1 μg/mL of GHB-D_6 as internal standard using the optimized GHB acquisition method previously described.

To evaluate matrix effects (ion suppression or enhancement), the ion source housing was equipped with a T-connector to allow post-column infusion of the analyte (equivalent to 2 and 10 ng/mg GHB). This produced a constant signal at the detector. Matrix effects were determined by injecting extracts from GHB-free hair samples and monitoring the detector response as reported elsewhere (19).

Results and Discussion

Instrument Optimization

Compound optimizations were performed by direct infusion of GHB and GHB-D_6 using ESI and APCI in both positive and

TABLE 1—*Parent and product ion transitions for GHB and GHB-D_6.*

		Parent Ion		Product Ion
ESI (+)	GHB	105.1	→	87.1
		105.1	→	68.9
	GHB-D_6	111.1	→	93.1
		111.1	→	73.0
APCI (+)	GHB	105.1	→	87.1
		105.1	→	45.1
	GHB-D_6	111.0	→	93.0
		111.0	→	49.0
ESI (−)	GHB	102.9	→	57.0
		102.9	→	85.0
	GHB-D_6	108.9	→	61.1
		108.9	→	90.0
APCI (−)	GHB	102.9	→	57.0
		102.9	→	85.1
	GHB-D_6	109.0	→	61.0
		109.0	→	90.0

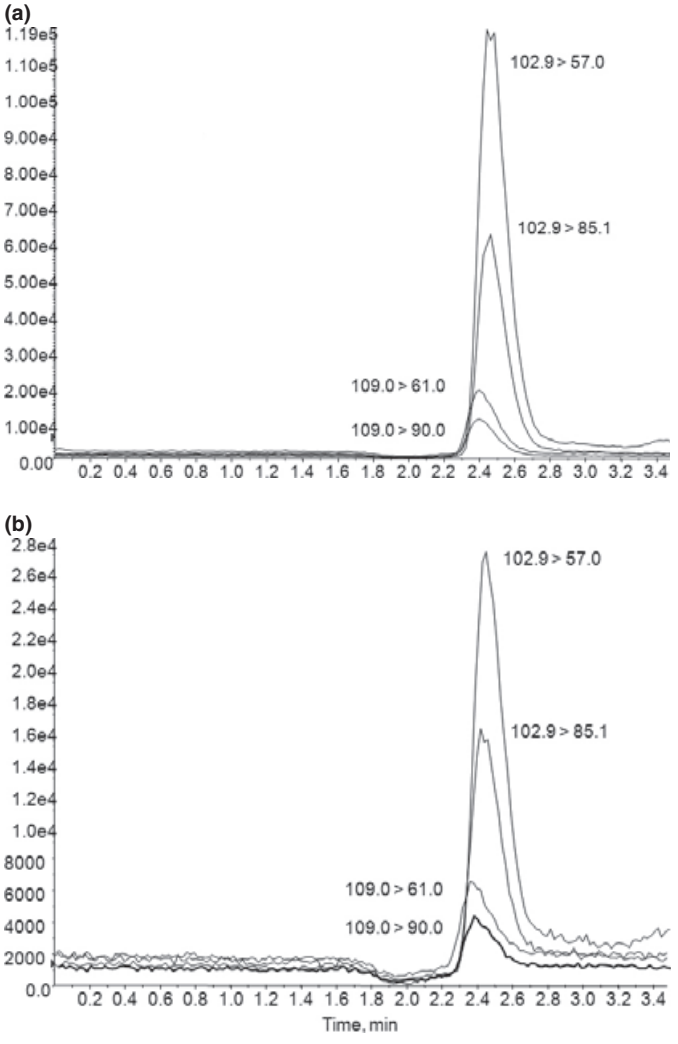

FIG. 1—*GHB (10 ng/mg) and GHB-D_6 (5 ng/mg) standard in (a) APCI (−) and (b) ESI (−) modes.*

negative modes. Parent and product ion transitions that were evaluated are summarized in Table 1. Following infusion, 0.5 mg/L standards were injected using the autosampler directly to the detector, bypassing the column, to optimize source parameters using FIA.

Unextracted samples (GHB standards), extracted standards (LLE), digested hair extracts with internal standard only, and digested hair extracts with internal standard and GHB were then evaluated under optimum source and ionization conditions for ESI (+), ESI (−), APCI (+), and APCI (−). Because of the overall higher response in negative mode and a significantly reduced background noise in APCI, APCI (−) was selected. To illustrate these differences, ion chromatograms for the direct injection of GHB (10 ng/mg) and GHB-D_6 (5 ng/mg), in both APCI (−) (A) and ESI (−) (B) are shown in Fig. 1.

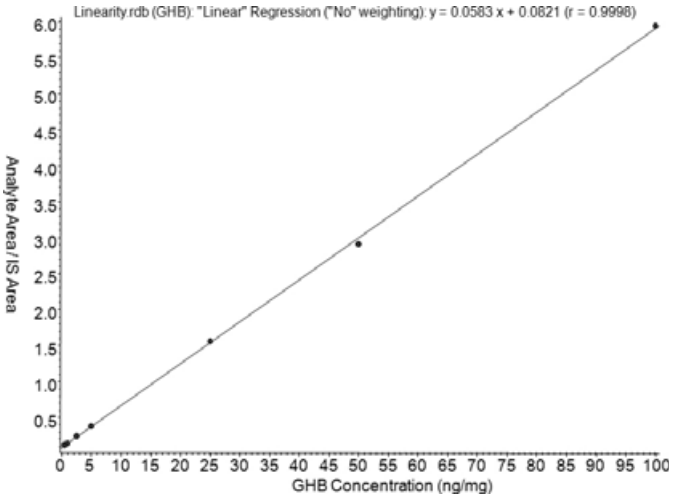

FIG. 2—*GHB calibrators in hair (0.2–100 ng/mg).*

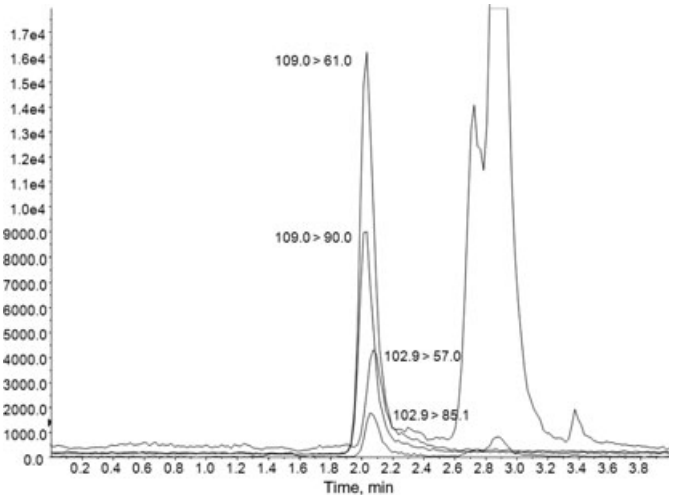

FIG. 3—*Hair extracts containing 0.2 ng/mg GHB (LOD). Hair samples were routinely fortified with 8 ng/mg GHB-D_6 (2.04 min).*

TABLE 2—*Ion transitions for α-hydroxybutyrate (AHB) and β-hydroxybutyrate (BHB).*

	Retention Time (min)	Parent Ion	Product Ions
AHB	3.65	102.9	57.0 85.1
BHB	2.88	103.0	58.8 58.2

Assay Optimization

Conversion of GHB and the internal standard to GBL and the corresponding deuterated lactone was evaluated in mobile phase. Comparison of ion intensities for each transition over 2 days showed no measurable decrease in stability for drug stored in mobile phase relative to deionized water. Samples were routinely stored at 4°C in mobile phase in between sampling and injection onto the LC/MS/MS. During the assay optimization, 25 mg of hair was routinely fortified with GHB (5 ng/mg and 20 ng/mg) and GHB-D_6. The optimum conditions for alkaline digestion were investigated using a variety of sodium hydroxide solution concentrations, digestion times, and temperatures. The quantity of 1 M sulfuric acid added to the cooled digest prior to the addition of ethyl acetate was also investigated. Optimum results in terms of abundance and cleanliness of the extract were obtained when the hair was digested at 75°C for 40 min, and 0.6 mL of 1 M sulfuric acid was used to neutralize and then acidify the digested matrix prior to the ethyl acetate extraction.

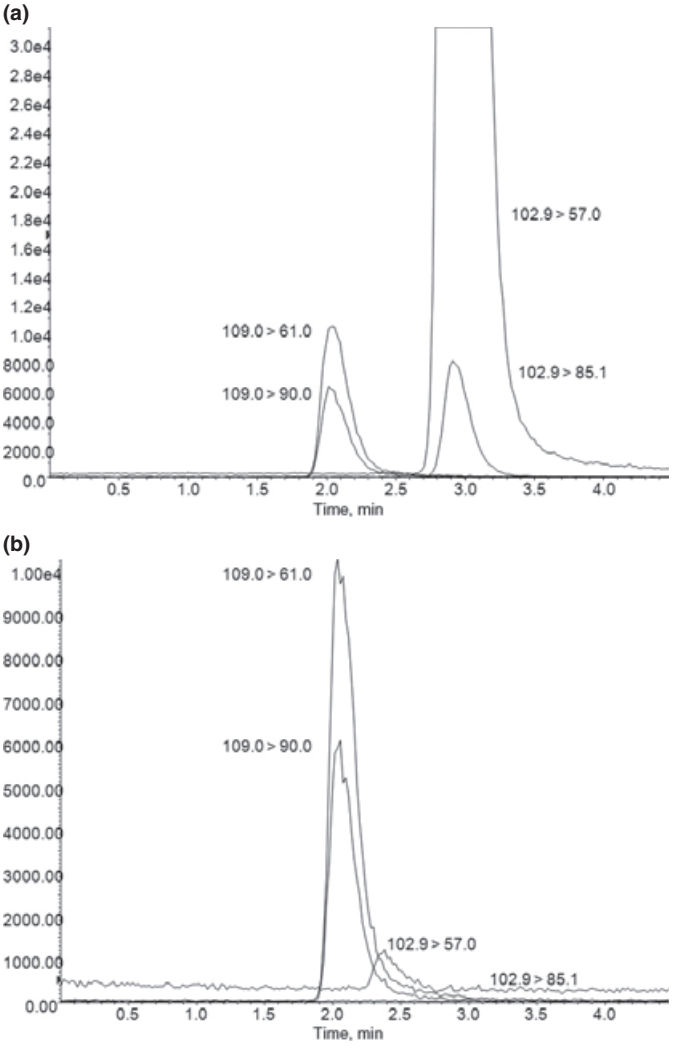

FIG. 4—*Injection of structural isomers (a) α-hydroxybutyrate and (b) β-hydroxybutyrate. Interferences were injected at 10 μg/mL in the presence of internal standard (1 μg/mL GHB-D_6) at 2.04 min.*

Assay Validation

Linearity was observed from 0.2 to 100 ng/mg (Fig. 2). Linear regression analysis indicated an R^2 value of 0.9996 in this range. The highest concentration tested was 100 ng/mg, and this was arbitrarily set as the limit of linearity. The assay was designed specifically for the quantitative analysis of endogenous GHB and even in cases of chronic GHB use or overdose, concentrations as high as 100 ng/mL have not been reported. Accuracy was determined to be 97% and 94% at 2 and 10 ng/mg, respectively, based on the mean of four determinations. Calculated concentrations for hair samples fortified with 2 and 10 ng/mg GHB were 1.93 ± 0.1 ng/mg and 9.41 ± 0.7 ng/mg corresponding with intra-assay CVs of 5.2% and 7.4%, respectively (n = 4). The LOD was determined to be 0.2 ng/mg, and the LOQ was 0.4 ng/mg. Figure 3 depicts the transitions for GHB-D_6 and GHB at the detection limit.

Potential interferences by the structural isomers AHB and BHB as well as GHB analogs GBL and 1,4 BD were also examined. α-Hydroxybutyric is a fruit acid found in juices, and β-hydroxybutyric acid is a metabolic byproduct associated with ketosis (20). Gamma-butyrolactone (GBL) is a compound found in floor stripper and stain remover, and 1,4 BD is an industrial solvent. Both substances are metabolized to GHB and are recreational drugs in their own right. To test for interference by these compounds, both compound-dependent and source-dependent

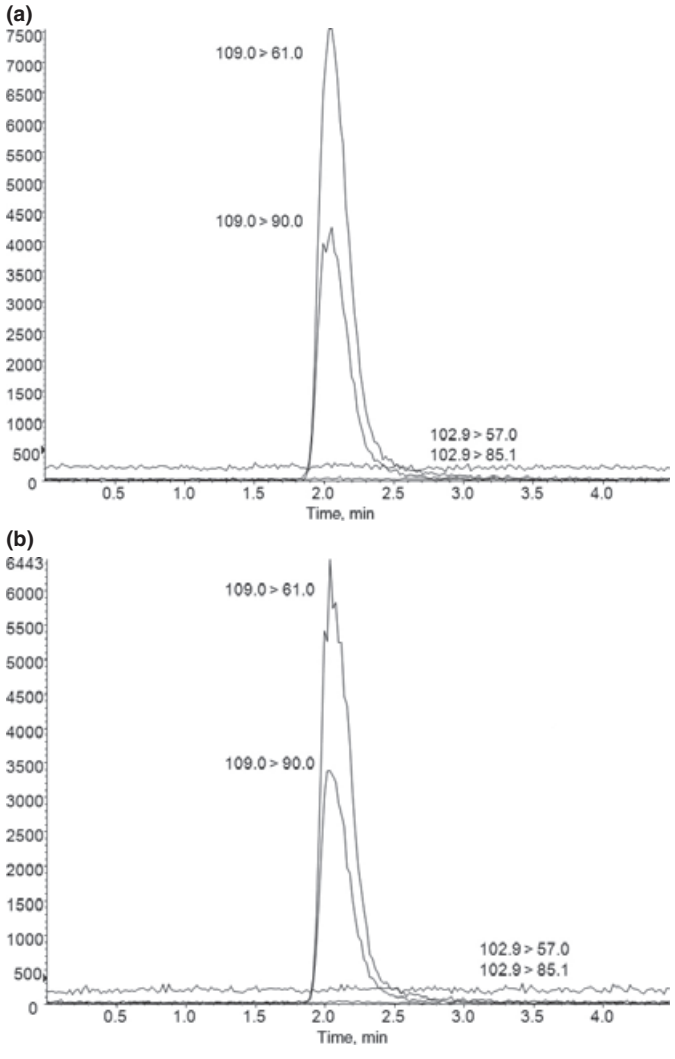

FIG. 5—*Injection of GHB analogs (a) 1,4-butanediol and (b) Gamma-butyrolactone. Interferences were injected at 10 μg/mL in the presence of internal standard (1 μg/mL GHB-D_6) at 2.04 min.*

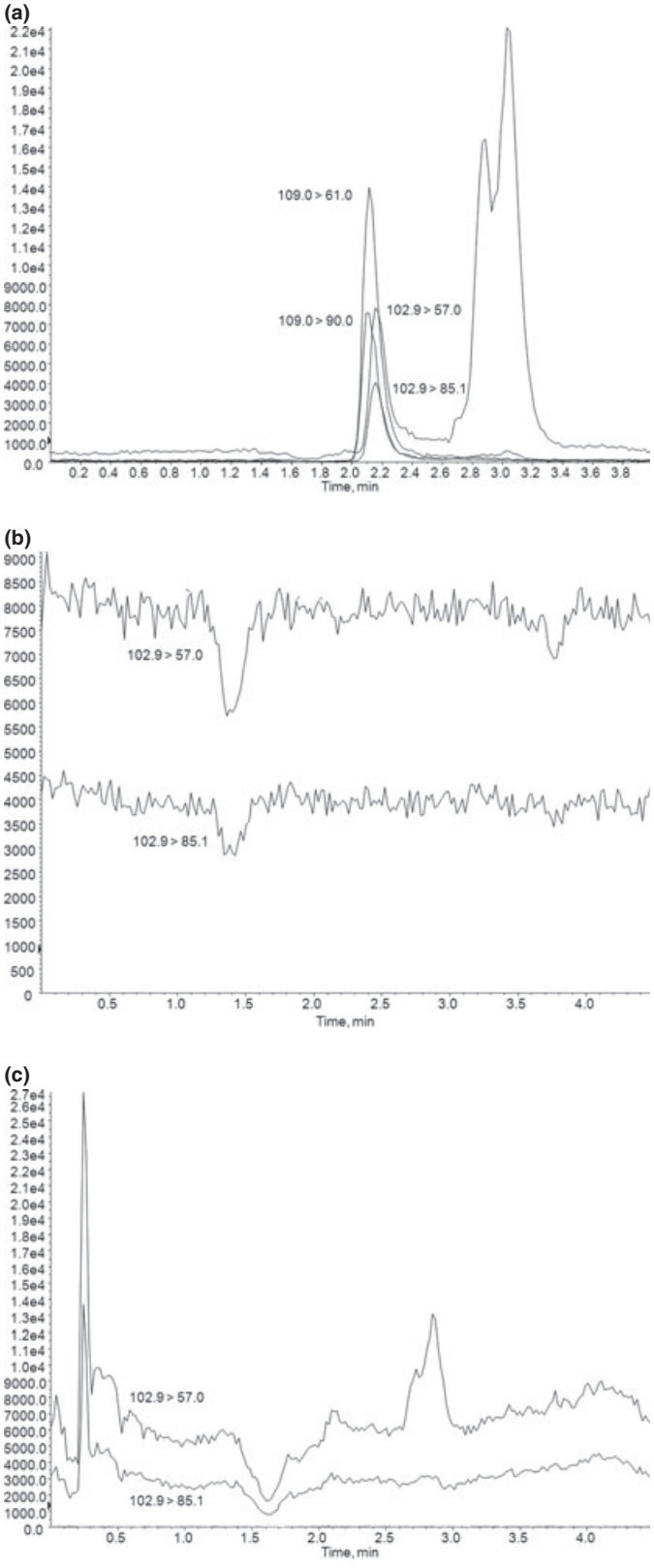

FIG. 6—*Ion suppression: (a) 2 ng/mg GHB standard (for comparison); (b) Mobile phase injection; (c) Injection of representative hair digest.*

parameters were optimized via infusion and FIA. Retention times and optimized transitions for the structural isomers AHB and BHB are given in Table 2. As expected, α-hydroxybutyric acid shared the same product ions as GHB, but the two compounds were completely resolved chromatographically; 3.65 and 2.04 min for AHB and GHB-D_6, respectively (Fig. 4*a*). Optimized product ions for β-hydroxybutyric acid were 58.8 and 58.2 as opposed to 57.0 and 85.1 for GHB. When injected using the optimized method for GHB, BHB showed the potential for interference with the 57.0 product ion of GHB. In practice, however, minimal interference was observed, even when injected at a very high concentration (10 μg/mL) (Fig. 4*b*). As expected, there were no interferences from either GBL or 1,4 BD because of the lower molecular weight of these prodrugs and the ability of Q1 to filter out these substances (Fig. 5).

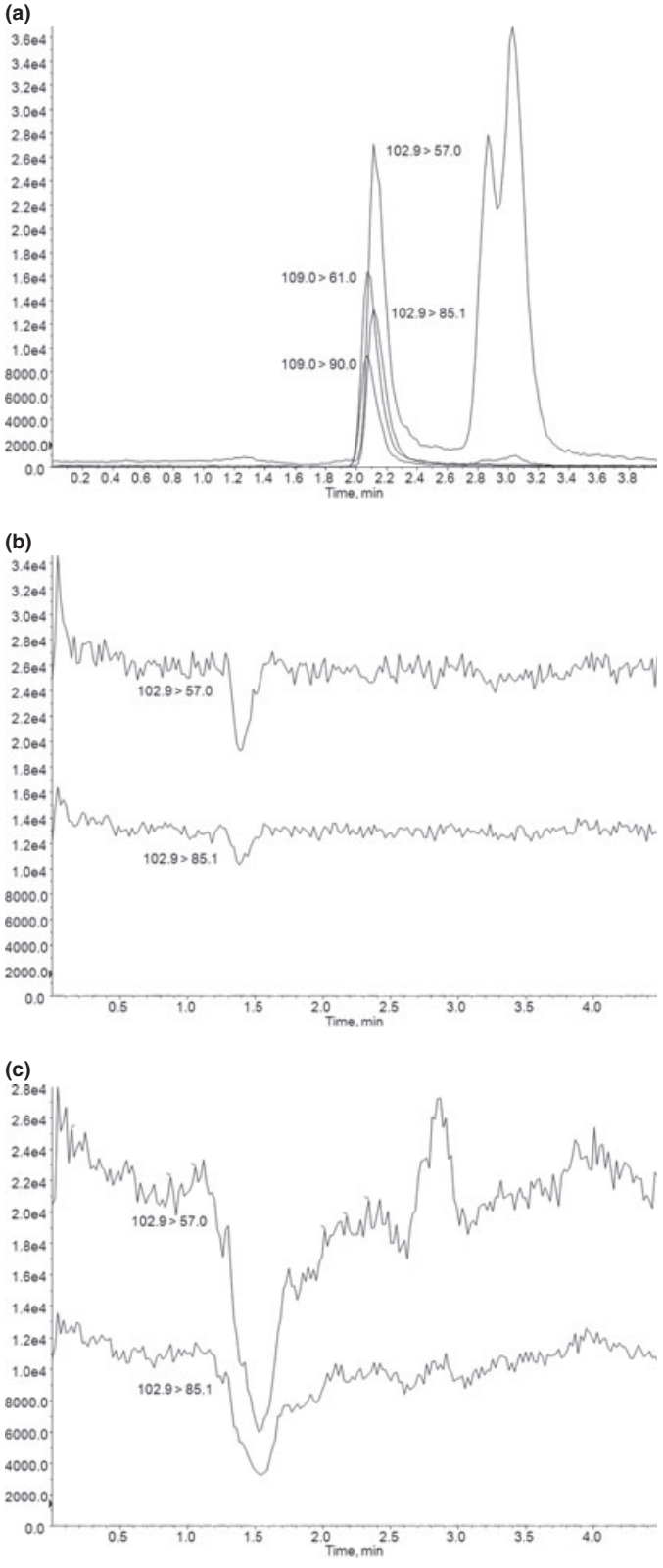

FIG. 7—*Ion suppression: (a) 10 ng/mg GHB standard (for comparison); (b) Mobile phase injection; (c) Injection of representative hair digest.*

Determination of matrix effect in a specimen that may contain detectable concentrations of the analyte poses a significant challenge. We sought to evaluate this using at least ten blank matrix extracts using the widely reported postcolumn infusion procedure (19). However, to evaluate the potential for ion suppression or enhancement, the matrix must be free from analyte. We were able to identify a total of six hair extracts with completely undetectable concentrations of GHB (less than the LOD of 0.2 ng/mg). Although this population was smaller than initially hoped, the extracts represented "true matrix" and more closely predict the potential for matrix interference than using a synthetic matrix. Ion suppression or enhancement was evaluated at 2 ng/mg (Fig. 6) and 10 ng/mg (Fig. 7). Of the six blank extracts identified, ion suppression or enhancement proved to be negligible at the retention time of GHB (2.04 min).

Conclusion

Using alkaline digestion of hair followed by liquid–liquid extraction and LC/MS/MS analysis, GHB was readily detected at 0.2 ng/mg, and quantitative analysis was possible at 0.4 ng/mg. APCI in the negative mode provided the greatest sensitivity and specificity for hair extracts, and optimized conditions were free from interferences from analogs and structural isomers. Overall assay performance in terms of precision, accuracy, linearity, detection, and quantitation limits is sufficient to allow for endogenous concentrations of GHB in hair to be fully investigated in an IRB approved future study.

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Additional information and reprint requests:
Sarah Kerrigan, Ph.D.
Forensic Science Program Director
College of Criminal Justice
Sam Houston State University
Box 2525
1003 Bowers Blvd
Huntsville, TX 77341
E-mail: sarah.kerrigan@shsu.edu