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TECHNICAL NOTE

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^{1}H NMR Analysis of GHB and GBL: Further Findings on the Interconversion and a Preliminary Report on the Analysis of GHB in Serum and Urine

ABSTRACT: A ^{1}H nuclear magnetic resonance (^{1}H NMR) method for the determination of gamma-hydroxybutyric acid (GHB) and gamma-hydroxybutyrolactone (GBL) in human serum and urine using spiked samples has been developed. The method gives linear responses (correlation coefficients of 0.99 or greater) over the concentration range 0.01 mg/mL to 4.0 mg/mL in urine and 0.3 mg/mL to 2.0 mg/mL in serum. No sample pretreatment is required. Studies of the chemical interconversion of GBL and GHB showed hydrolysis of GBL to be rapid at pH 11.54, slower and less complete (30% hydrolysis) at pH 2.54 and slowest at pH 7.0, reaching 30% hydrolysis in about 40 days. No esterification of GHB was observed at any pH.

KEYWORDS: forensic science, gamma-butyrolactone (GBL), gamma-hydroxybutyric acid (GHB), interconversion, NMR, serum, urine

An increasingly popular substance of abuse, Gamma Hydroxybutyric Acid (GHB, Fig. 1), is becoming apparent on the streets in a variety of forms (1). Although GHB is an endogenous substance naturally found in the brain, many people still find reason to abuse it, such as euphoria, and its hallucinogenic properties as well as its role as a sexual assault drug (2). GHB is often recognized on the streets as Liquid Ecstasy or Liquid X, as well as other names. It is a popular substance often found at "rave parties," upscale "smart-drink" nightclubs and confiscated in "spring water" bottles or disguised as mouthwash. Originally, it was marketed in health food stores, as a growth hormone releasing agent to stimulate muscle growth. GHB was later taken off the shelves and designated in 2000 by the Drug Enforcement Agency (DEA) as a Schedule I substance of the Controlled Substance Act (CSA).

Gamma-butyrolactone (GBL, Fig. 1) is a corresponding cyclic ester of GHB and can easily hydrolyze back to GHB in aqueous solutions (3). Thus, due to the GBL-GHB chemical interconversion, it holds important legal and forensic implications. GBL is classified only as a DEA List I chemical; however, if ingested, it could be treated as a Schedule I substance under Federal law. In response, many commercial GBL products, which are often available even in local convenience stores, have emerged in recent years. Also commonly attainable in industrial solvents, GBL is also used to produce pesticides, pharmaceuticals, and other products. Therefore, it is important to understand the interconversion process in order to develop efficient and accurate analytical methods.

Gas chromatography analyses such as GC/MS are by far the most popular means for measurement of GHB in forensic samples (4,5). Although sensitive, these methods require the chemical conversion of GHB into the volatile GBL or silyl derivatives. Other techniques include Fourier Transform infrared spectroscopy (FTIR) (6), ion mobility spectrometry (7), and high-performance liquid chromatography (HPLC/UV, HPLC/MS) (8). Detection with these methods still suffers from requiring additional steps such as extraction and the use of buffered solutions. Thus, they are not particularly suitable for investigating the GBL-GHB equilibrium. Bravo et al. (8) have recently described a semi-quantitative "dipstick" test sensitive to about 0.05 mg GHB/mL. The assay is based on GHB dehydrogenase (GHB-DH/NAD^+) oxidation of GHB followed by diphorase-mediated colorimetric analysis.

Nuclear magnetic resonance spectroscopy (NMR) is a recognized tool for the direct detection of a wide range of small endogenous or exogenous molecules in complex biofluids such as urine and serum ("metabonomics") (9). Therefore, NMR is ideal for direct monitoring of the GBL-GHB equilibrium, without the issues associated with extraction, derivatization, chemical compatibility, and solvent effect. Chew et al. (10) have recently employed 2D NMR (COSY, HETCOR) to assign the ^{1}H and ^{13}C signals of GHB in D_2O and reported a highly linear standardization curve ranging 5–100 mg/mL of GHB.

Here, we report the effective use of ^{1}H NMR for quantifying the relative amount of GHB and GBL in various buffered solutions. We also describe spike experiments involving human urine and serum samples to determine the detection limit for GHB.

Materials and Methods

Following DEA guidelines, GHB sodium salt and GBL were obtained from Aldrich Inc. (Milwaukee, WI). Deionized water was obtained from a Millipore Milli-Q filtration system.

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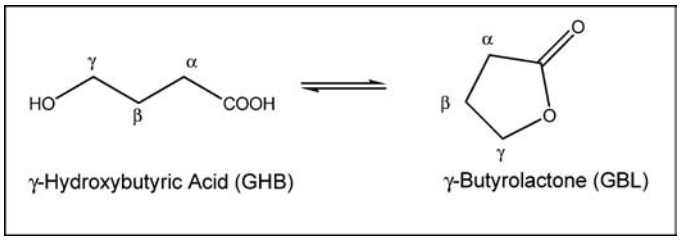

FIG. 1—*The structures and nomenclature of γ-hydroxybutyric acid (GHB) and γ-butyrolactone (GBL) and their chemical interconversion.*

Urine samples were obtained using an IRB protocol approved for obtaining random healthy volunteers. Urine specimens were collected from eight individuals asked to join the study in a random anonymous fashion, to prevent any sort of bias. Normal pooled human serum sample, from a minimum of 6 units, was purchased from Interstate Blood Bank, Inc. (Memphis, TN). Both the urine and serum samples were pooled into a single container and stored at 4°C until time of experimentation. Acquisition of urine and serum samples was done in accordance with the Helsinki Declaration of 1983.

The GBL-GHB Interconversion in Buffered and Spiked Solutions

Potassium phosphate monobasic/dibasic solution (1 M) was prepared and then adjusted to pH 11.54, 7.00, 2.75 using diluted HCl and NaOH. For preparation of GHB or GBL in the various phosphate buffers, equal volumes of the GHB or GBL stock solution and phosphate buffer were added to NMR tubes and vortexed. The final GHB and GBL concentrations were 6 mg/mL in 0.5 M buffer. Using a similar procedure, an 11 mg/mL solution of GHB or GBL and tap water (pH 5.85) was made up in a NMR tube and vortexed.

Commercial products were also studied, using a 12 oz can of Sprite® Lemon-Lime soda. The beverage pH was acidic, ranging from 3.13 to 3.25. GHB and GBL were each mixed with Sprite in NMR tubes, with a concentration of 6 mg/mL. All mixtures were brought up to a final volume of 1 mL using the appropriate solvent. Upon vortexing the mixture, all solutions were stored under ambient conditions (22°C).

Urine and Serum Spike Studies

For the first set of urine experiments, a series of concentrations were made up from 0.0 (control), 0.01, 0.05, 0.10, 0.20, 0.30, 0.40, 0.50, 0.75, 1.0 mg/mL. This was done to determine the range of detection for the GHB samples. The second experiment consisted of using concentrations of 0.0 (control), 0.20, 0.40, 0.60, 0.80, 1.0, 2.0, 3.0, 4.0 mg/mL. Finally, seven subsamples were prepared using 0.3 mg/mL and used to determine the Method Detection Limit.

For the serum experiment, 7 samples were made up using the following concentrations: 0.0 (control), 0.30, 0.50, 0.70, 0.90, 1.0, 2.0 mg/mL to determine the range of detection. Subsequently, seven subsamples at a concentration of 0.5 mg/mL were made up to determine the Method Detection Limit.

NMR Analysis

^{1}H NMR spectroscopy was employed to monitor GBL-GHB chemical interconversion in aqueous solution and to analyze the levels of GHB in the urine and blood samples. The samples were prepared from a pooled source by spiking the solution, and were analyzed directly without pretreatment or extraction. Analyses were performed within 8 hours of sample preparation to minimize possible hydrolysis.

^{1}H NMR spectra were acquired on a Bruker AM 300 MHz spectrometer, with presaturation of the water signal. The principle features of the NMR analysis include a presaturation time of 3 s, spectral width of 6 kHz, acquisition time of 1 s and a tip angle of 80°. A 0.20 Hz exponential multiplication is applied to the data, averaging 64 transient results, in an overall analysis time of 5 min.

Calibration Curve Solutions

A concentration gradient consisting of 10 solutions each with a volume of 0.8 mL were prepared starting from 0 (control), 0.014, 0.067, 0.14, 0.26, 0.38, 0.53, 0.66, 0.95, 1.20 mg/mL. These concentrations were utilized for the first set of calibration curve tests. Subsequently, another set of 8 solutions, each at 0.8 mL, were prepared starting with 0.025, 0.50, 0.75, 1.00, 2.00, 3.00, 4.00, 5.00 mg/mL.

Results and Discussion

The Interconversion of GBL and GHB in Various pH Conditions

We conducted experiments, whereby GBL was added into solutions of different pH's of 11.54, 7.00, 2.75. The solutions were mixed and stored under ambient temperatures and analyzed for GHB and GBL content using ^{1}H NMR. The experiments continued for about 70 days with measurements taking place at various time intervals. A typical ^{1}H NMR spectrum of GHB/GBL mixture in buffered solution is shown in Fig. 2. The signal assignments were made, based on homonuclear decoupling experiments, and confirmed by the earlier 1D and 2D NMR results (10,11). After baseline correction, the areas for H_γ of GHB and H_α of GBL were integrated, and quantified using the equation that determines the proportion of GHB by [GHB/ (GBL + GHB)] × 100%.

The time dependent GBL to GHB conversions at three prototypic pH's (7.00, 11.54, 2.75) are shown in Fig. 3. The hydrolysis of GBL at pH 7.00 was slow initially and progressed linearly, but reached an equilibrium of (70% GBL:30% GHB) in about 40 days. No consistent plateau was reached during the 60 days, although the beginning of one was forming. The hydrolysis of GBL in tap water at pH 5.85 was also carried out. The overall conversion was a little less (77% GBL:23%GHB) but showed a similar hydrolysis pattern (data not shown).

At pH 11.54, the hydrolysis of GBL (Fig. 3) proceeded rapidly, giving rise to an equal mixture of GBL:GHB in about 30 min. The initial plateau (58% GHB) was reached in 48 hours, and an equilibrium ratio of (15% GBL:85% GHB) was obtained after 67 days of storage. However, the hydrolysis continued, and no clearly defined equilibrium plateau was found.

The hydrolysis of GBL at pH 2.75 proceeded at a slower rate and was less than pH 11.54, but a consistent equilibrium plateau was attained after 20 days of storage (71% GBL:29% GHB). The extent of hydrolysis of GBL at various pH's in our work is largely comparable to those obtained from HPLC (3). The clear-cut plateau obtained from the time dependent % GHB profile of the acidic (pH 2.75) solution is contrasted with that of the basic counterpart (pH 11.45) that exhibited progressive hydrolysis throughout the experiment. This confirms the observation of Ciolino et al. (3) who suggested the formation of the stable GHB anion or salt in basic pH prevents reesterification back to GBL. Under acidic conditions, however, hydrolysis/reesterification processes are competing to help reach a stable equilibrium endpoint.

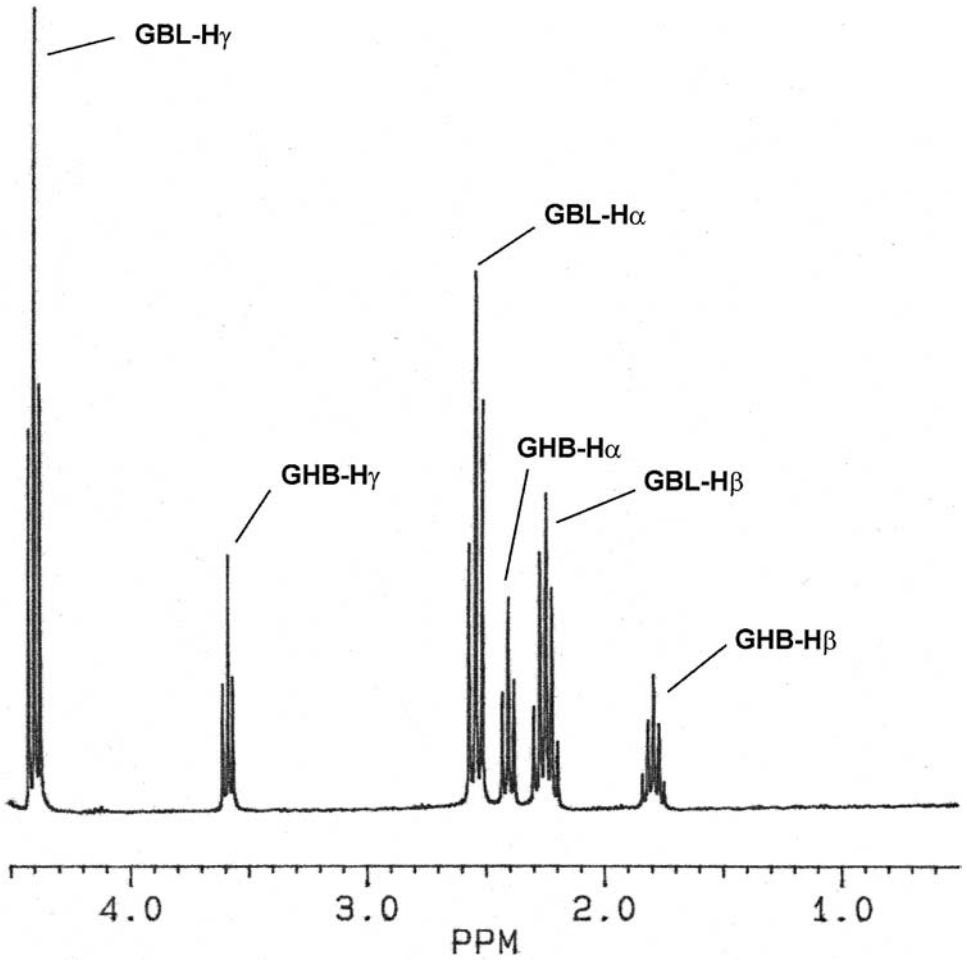

FIG. 2—*Typical ^{1}H NMR spectrum showing partial hydrolysis of GBL to GHB (pH 2.75, 50 days). See Figure 1 for structures and nomenclatures.*

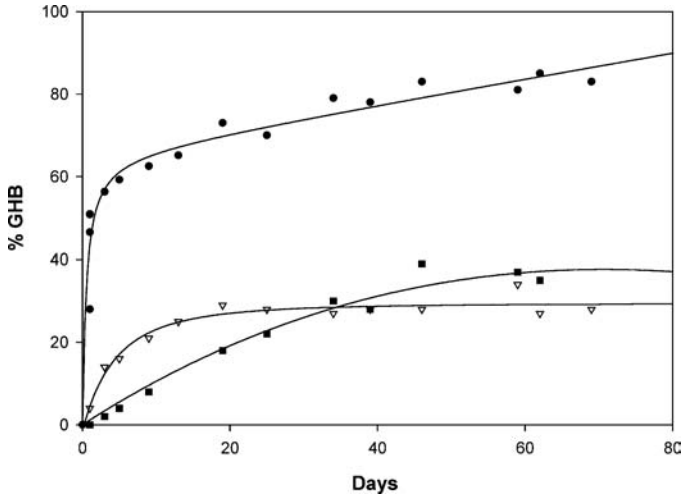

FIG. 3—*pH-Dependent hydrolysis of GBL to GHB as a function of time.* ● *pH 11.54;* ▽ *pH 2.75;* ■ *pH 7.00.*

A similar set of experiments was conducted to monitor the conversion of GHB to GBL. In the three pH's employed, there was no esterification apparent throughout the 70 day observation period. This is surprising because the above-cited paper (3) indicated a facile conversion of GHB to GBL in pH 2.0, reaching an equilibrium of about 2:1 mixture of GBL:GHB; no esterification occurred in the pH range 4.0 to 6.4 in the first 10 days but slowly reached varying degrees of equilibrium at the end of 220 days. The reason for this discrepancy is not clear but is possibly due to the conditions at which the samples were analyzed: i.e., Ciolino et al. (3) have employed a HPLC mobile system consisting of acidic phosphate buffer, whereas our NMR method represents a direct detection.

Commercial Product Studies: Interconversion of GBL and GHB

The commercial product used was a nonalcoholic lemon lime carbonated soda (Sprite®) with a pH of 3.13 for the GHB study and a pH of 3.25 for the GBL study. The measurement took place over a 67-day period in which the samples were stored in at room temperature.

The hydrolysis of GBL in this solution was relatively slow and did not begin until 7 days after the start of the experiment. From then on, the conversion took place gradually. The experiment was terminated at 67 days with a ratio of (71% GBL:29% GHB) (data not shown). As in the case of buffered solutions, no esterification of GHB to GBL took place during the 67-day observation period.

Urine and Serum Studies

Urine and serum samples were spiked with known amount of GHB (see Methods) and then analyzed by ^{1}H NMR for detection

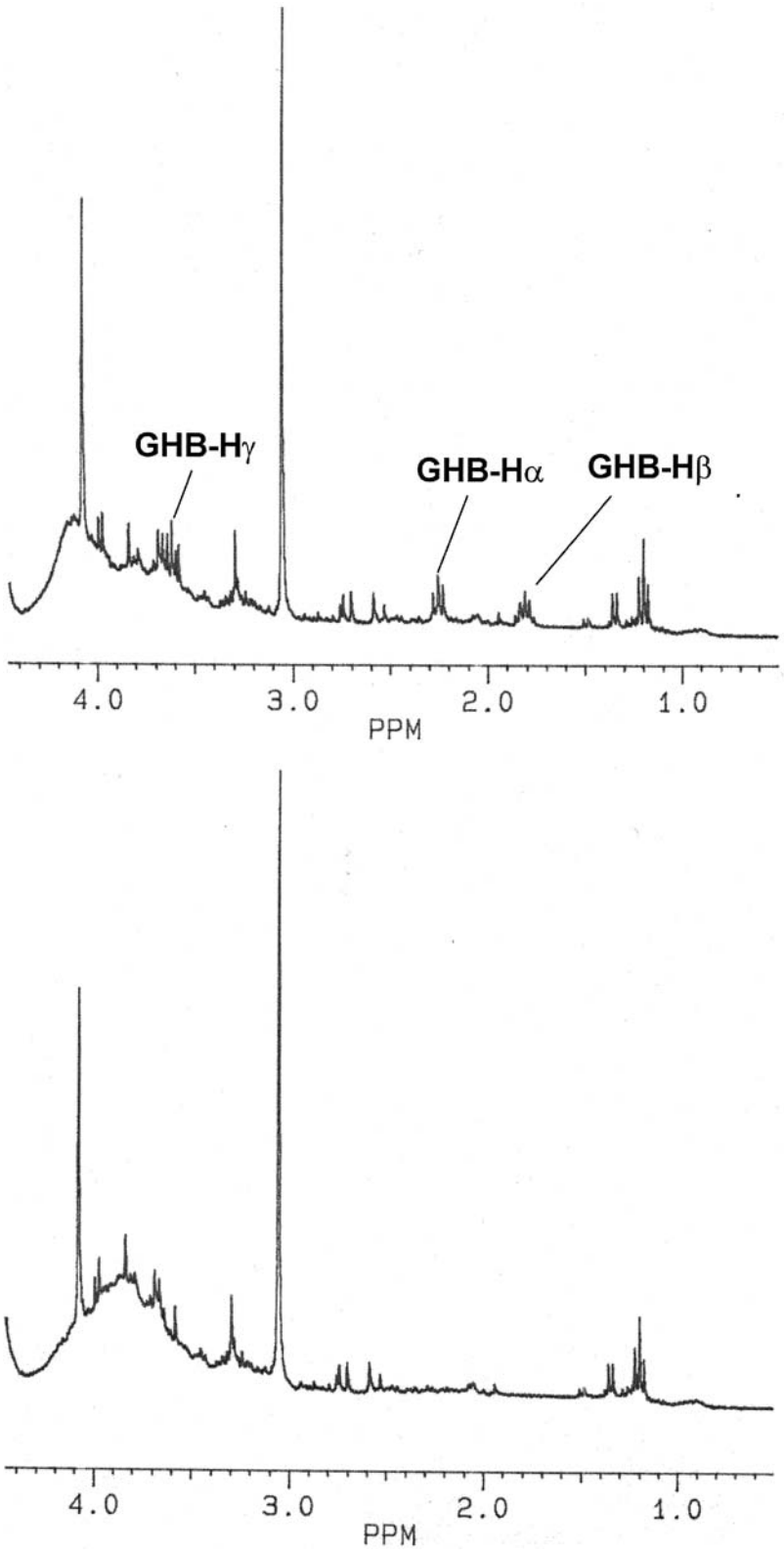

FIG. 4—*Typical ^{1}H NMR spectra of GHB (0.2 mg/mL) in urine (upper trace) and negative control (lower trace).*

of GHB levels. Figures 4 and 5 shows typical ^{1}H NMR spectra of the urine and serum samples and their corresponding controls, respectively. Figures 6 and 7 shows calibration curve results for GHB in urine and serum, respectively. The method produces a linear response over the range studied with correlation coefficients of 0.99 or greater in both matrices.

Method detection limits were determined based on the precision of the analysis at the limit of quantitation, according to Federal guidelines (12). In urine, analysis of seven independent samples spiked at a concentration of 0.3 mg/mL showed a relative standard deviation of 2%. The method detection limit is 0.05 mg/mL and the limit of quantitation is 0.3 mg/mL.

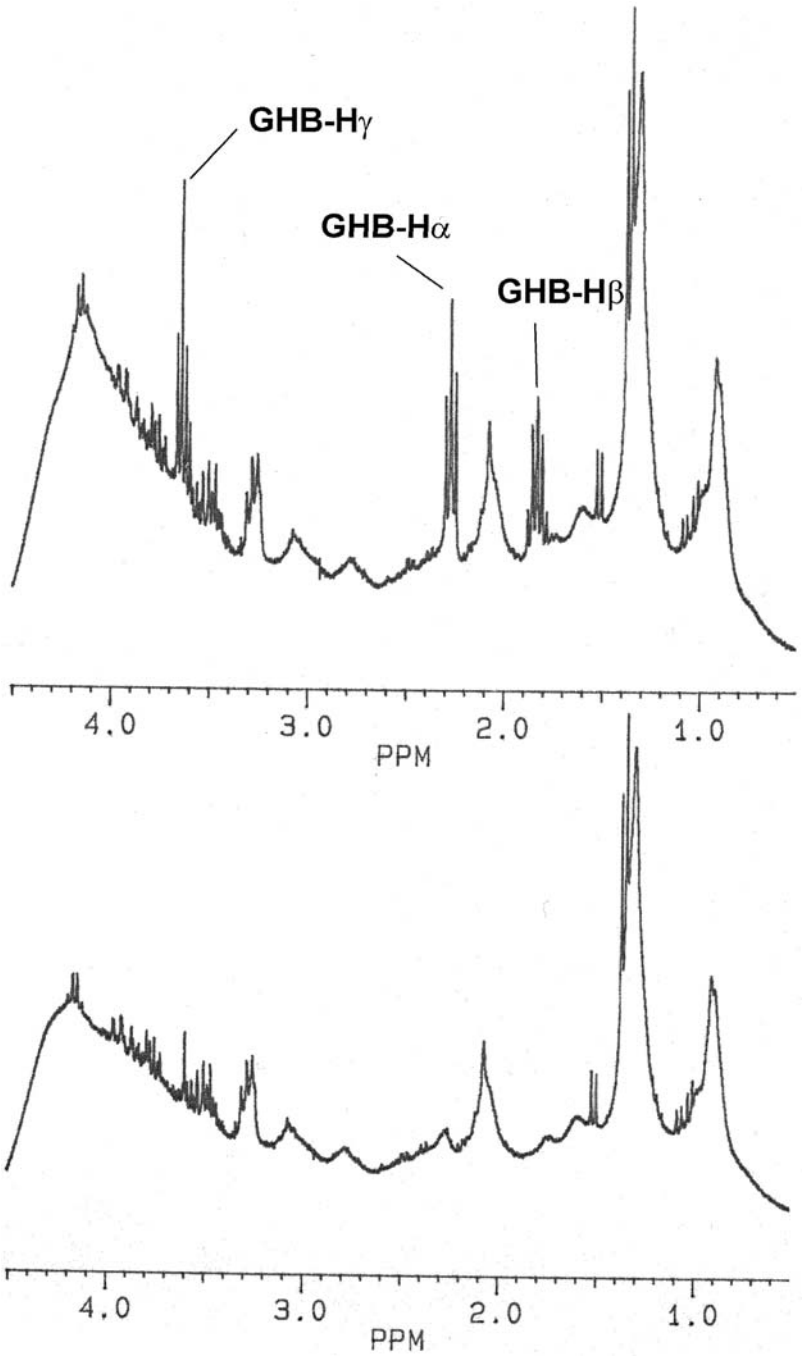

FIG. 5—*Typical ^{1}H NMR spectra of GHB (0.3 mg/mL) in serum (upper trace) and negative control (lower trace).*

In blood serum, analysis of seven independent samples spiked at a concentration of 0.5 mg/mL showed a relative standard deviation of 5%. The method detection limit is 0.1 mg/mL and the limit of quantitation is 0.5 mg/mL.

Conclusions

We have demonstrated the utility of ^{1}H NMR for analyzing the GHB/GBL chemical interconversion as well as quantification of GHB in biological fluids such as urine and blood serum. NMR has an advantage over other methods, because samples can be analyzed rapidly and directly without the need of extra sample cleanup, extraction, or chemical derivatization. The integrated NMR signal displayed linear response (correlation coefficients of 0.99 or greater) across the concentration ranges, studied in both urine (0.01 mg/mL to 4.0 mg/mL) and serum (0.3 mg/mL to 2.0 mg/mL) matrices. ^{1}H NMR allows detection of GHB when concentrations are at low levels in complex biological media and serves as a viable alternative method. Although not as sensitive as GC/MS methods, the sensitivity of the ^{1}H NMR method is comparable to that of a recently developed enzyme-based "dip stick" assay (ca. 0.05 mg/mL) (8). The ^{1}H NMR method presented here, however, has been developed using samples to which pure GHB has been added. For full clinical use, the ^{1}H NMR method must be validated with authentic samples and specificity studies should be performed.

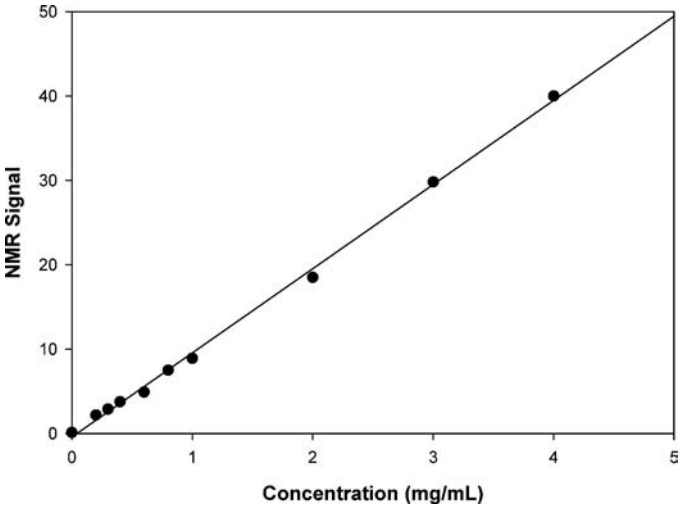

FIG. 6—*Standard curve (r = 0.991) of GHB in urine samples.*

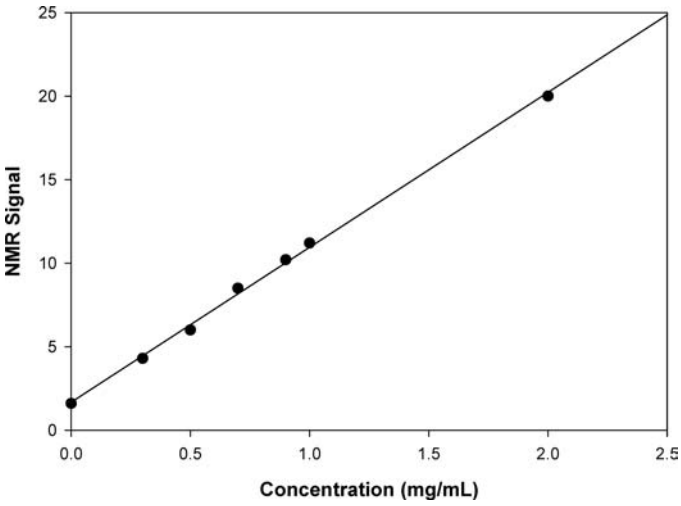

FIG. 7—*Standard curve (r = 0.998) of GHB in serum samples.*

Acknowledgments

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