TECHNICAL NOTE

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Determination of Gamma-hydroxybutyrate in Water and Human Urine by Solid Phase Microextraction-Gas Chromatography/Quadrupole Ion Trap Spectrometry*

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ABSTRACT: A simple method of detection was developed for gamma-hydroxybutyrate (GHB). The method involves the derivatization of GHB using a hexyl-chloroformate procedure in aqueous media (such as water or urine), extraction of the derivatization product directly from the sample using solid-phase microextraction, and subsequent separation and detection with gas chromatography quadrupole ion trap mass spectrometry. The deuterated form of GHB (GHB-D_6) is used as an internal standard for quantitation. The method was linear for GHB-spiked pure water samples from 2 to 150 μg/mL GHB with a detection limit of 0.2 μg/mL. Spiked urine samples showed linearity from 5 to 500 μg/mL GHB with a detection limit of 2 μg/mL. The SPME-GC/MS method is applied to actual case samples, and the results are compared to those values obtained using a conventional GC/MS method. Sensitivity and linearity are comparable to those seen using traditional methods of separation, yet the SPME method is superior due to the simplicity, speed of analysis, reduction in solvent waste, and ability to differentiate between GHB and gamma-butyrolactone (GBL).

KEYWORDS: forensic science, gas chromatography/mass spectrometry, solid phase microextraction, quadrupole ion trap, gamma-hydroxybutyrate, hexyl-chloroformate derivatization

Gamma-hydroxybutyrate (GHB), which functions as a neurotransmitter and neuromodulator, is a natural metabolite of gamma-aminobutyric acid found in the mammalian brain (1). Over 95% of an oral dose of GHB is metabolized into carbon dioxide and water, leaving less than 5% to be detected in excreted urine, and it is undetectable after twelve hours (2,3). GHB has been used medically in Europe for many purposes, such as an anesthetic and in the treatment of sleep disorders and alcoholism, because of its ability to induce sleep (4,5). However, GHB has also been used for illicit purposes, such as recreational drug use and sexual assault, for this same sleep-inducing effect. GHB has many severe negative side affects, i.e., seizures, nausea, vomiting, dizziness, and disorientation, which are experienced at higher dosages (6). As a result, the U.S. Food and Drug Administration banned the sale of this drug in 1990, except for the clinical treatment of narcolepsy. Because of the increasing incidence of GHB use in young adults, the need for rapid, sensitive means of detecting GHB has become an important analytical objective, especially because GHB is odorless, tasteless, and frequently renders the user unresponsive.

Currently, the most common methods of detection for GHB typically involve the use of high performance liquid chromatography (HPLC) (7,8), gas chromatography (GC) (9), gas chromatography-mass spectrometry (GC/MS) (10–14), and HPLC/MS (8). To use these detection methods for biological samples, such as urine, sample pretreatment is necessary such as using one of many multi-step extraction methods for GC/MS analysis. GHB (a small, polar molecule) must first be derivatized. Many sample preparation methods involve conversion of GHB to its lactone form, gamma-butyrolactone (GBL) using acid catalysis (7,10–13). Followed by liquid-liquid extraction, GBL then may be detected (13). Also, GBL may be hydrolyzed and then derivatized, typically with bis(trimethyl-silyl)-trifluoroacetamide (BSTFA) (7,11,14). Subsequently, the derivatized form of GHB is detected. Others have reported the extraction of GHB or derivatized GHB using solid phase extraction (SPE) techniques prior to detection with GC/MS (9,14). In the present study, GHB is derivatized directly in the aqueous sample aliquot using a chloroformate derivatization procedure. The reaction mechanism for this derivatization is shown in Fig. 1. The derivatized form of GHB is directly extracted from the aqueous sample using a solid-phase microextraction fiber prior to detection with GC/MS. This technique allows for the detection of GHB without the formation of GBL. Furthermore, GBL is not isolated nor detected using this method.

Solid-phase microextraction (SPME) has recently proven to be a fast and simple method for analyte extraction, requiring only one solvent-free step for the preconcentration of a compound (15–17). In contrast, the conventional extraction methods used previously consist of multi-step techniques that involve excess organic solvents and preconcentration steps. The use of SPME-GC/MS has many advantages over the conventional extraction methods, including saving time and eliminating solvent waste. SPME uses a

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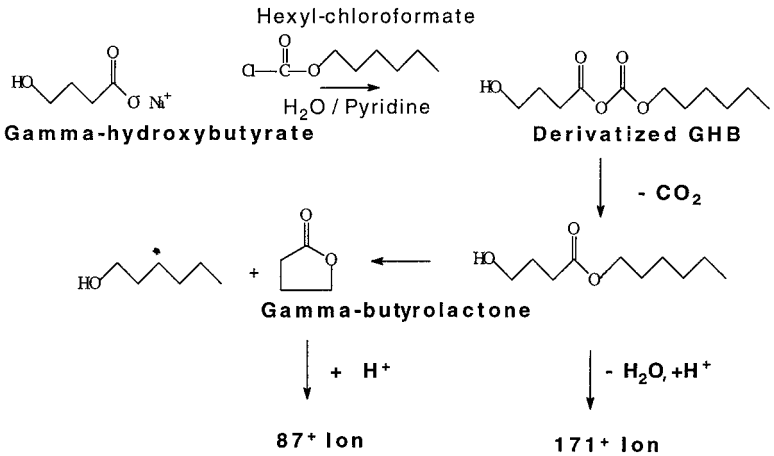

FIG. 1—*Chloroformate derivatization reaction.*

fused silica fiber that has been coated with a stationary phase. This fiber is submerged into an aqueous solution containing the compound or compounds of interest. The organic analytes partition into the stationary phase and equilibrium extraction conditions may be achieved. The SPME fiber is then removed from the solution and placed directly into the injection port of a GC or HPLC instrument for separation and detection of absorbed analytes (17). In this study, SPME is used in conjunction with gas chromatography and quadrupole ion trap mass spectrometry (GC/QIT-MS) for the determination of gamma-hydroxybutyrate (GHB) in both spiked water and urine samples.

Experimental

Materials

Gamma-hydroxybutyrate-sodium salt, gamma-butyrolactone, pyridine, potassium carbonate, dimethyl-aminopyridine, phosphate buffer solution (pH 7), and ethyl-, propyl-, butyl-, and hexyl-chloroformates were purchased from Aldrich (Milwaukee, WI). The internal standard, GHB-d_6 in methanol (100 μg/mL), was obtained from Radian International (Austin, TX). GHB concentrated stock solution was prepared in deionized water (1 mg/mL). Urine samples for spiking purposes were collected from a person who had no exposure to GHB. Aliquots of case urine specimens known positive for GHB were obtained from The Travis County Medical Examiner's Office (Austin, TX).

SPME Apparatus and Methods

The SPME holder for manual injections was obtained from Supelco (Bellefonte, PA). Three different fibers, a 100 μm polydimethylsiloxane (PDMS), a 65 μm polydimethylsiloxane-divinylbenzene (PDMS-DVB), and a 65 μm carbowax-divinylbenzene (CW-DVB) were obtained from Supelco. The PDMS fiber was chosen for use throughout the study based on preliminary experiments. All extractions were performed in 4 mL glass sample vials with Teflon caps, through which a small hole was drilled to allow the fiber to be inserted into the sample vial, and the fiber was exposed to the sample solution for an extraction period of ten minutes.

Sample solutions for derivatization were made by spiking either deionized water or urine with various amounts of a more concentrated GHB solution or GBL solution. It was necessary to use GHB-d_6 as an internal standard for the construction of calibration curves and quantitation. The spiked solutions (1 mL) were combined with 10 μg of internal standard (when used for quantitation) and derivatized by adding 40 μL of base and 24 μL of chloroformate derivatization agent. This reaction mixture was then sonicated for a period of 5 min at 40°C to aid the reaction. The SPME extraction solutions were a total volume of 3.5 mL, prepared by diluting 0.5 mL of the derivatized aqueous sample solution with 2.0 mL of deionized water and 1.0 mL of pH 7 buffer. The sample extraction solution was heated to 40°C and rapidly stirred via a Teflon coated magnetic stir bar during each extraction. Rapidly retracting and reexposing the fiber to the solution periodically removed air bubbles, which form on the fiber during extraction and affect extraction. After extraction, the SPME apparatus was transferred to the injection port of the gas chromatograph for a desorption period of 12 min onto the GC column. The injection port remained at the constant temperature of 270°C to desorb the analyte without damaging the fiber.

For comparison to the hexylchloroformate/SPME method, a conventional GC/MS procedure for GHB analysis was utilized with modifications (13). Briefly, 1 mL of urine was acidified with 2 mL 0.5 N HCl and heated for 20 min at 80°C to convert GHB to GBL. A 1 mL volume of 1.5 N phosphate buffer (pH 7) followed by 1.9 mL of 0.5 N NaOH was added to the cooled solutions. After addition of 30 μL of 1 mg/mL gamma-valerolactone as an internal standard, the samples were then extracted with 5 mL of *n*-butyl chloride and subsequently collecting the *n*-butyl chloride layer and discarding the aqueous phase. The organic phase was back extracted with 5 mL of 1 N HCl. The aqueous layer was collected, made basic with NH_4OH, and extracted with 60 μL of chloroform. A 2 μL volume was injected for GC-MS analysis. Analysis by this conventional method was performed in duplicate for case specimens.

The optimal derivatization base was determined by keeping all concentrations and parameters constant and varying the base added prior to derivatization. Likewise, the best derivatization agent was determined by keeping all experimental parameters constant while only changing the alkyl chloroformate used for derivatization. Salt effect was examined by keeping the concentration of GHB constant while changing the percentage of saturated sodium chloride in the solution used to dilute the postderivatization reaction prior to ex-

traction. The carryover percentage of the PDMS SPME fiber was determined by performing varied extraction periods and then subsequent desorptions until the compounds reached a lower limit of carryover. Linear range was determined by extracting derivatized GHB and GHB-d_6 from both spiked water and urine samples with concentrations of 2 to 150 μg/mL and 5 to 500 μg/mL, respectively.

Instrumentation

All experiments were performed on a Varian Saturn 4D gas chromatograph-mass spectrometer with a DB5-MS column. The GC oven was programmed to ramp from 60°C to 270°C at 9°C per min totaling 23.33 min. A Jade Valve injector (Alltech Associates, Inc., Deerfield, IL) was kept at a constant temperature of 270°C throughout the GC program. Helium was used as the carrier gas and set at an injector head pressure of 12 psi.

The Saturn GC/MS contains a quadrupole ion trap mass spectrometer detector. All experiments were performed in the electron ionization (EI) mode with the following parameters: 25 μA filament current, AGC target of 65 100 counts and electron multiplier voltage of 2500 V. The mass range scanned for all experiments was 83 to 270 amu. Selected ion monitoring (SIM) detection was used for more accurate quantitation for both water and urine matrix samples. The ions monitored were 87^+ and 171^+ for GHB and 93^+ and 177^+ for GHB-d_6. Proposed structures of these ions are shown in Fig. 1. The 171^+ and 177^+ ions are formed by decarboxylation of the derivatization product, and the 87^+ and 93^+ ions are formed by thermal degradation of the hexyl esters, 171^+ and 177^+.

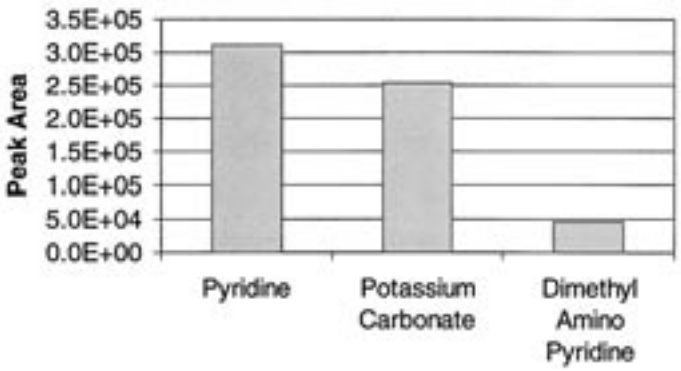

FIG. 2—*Derivatization base comparison.*

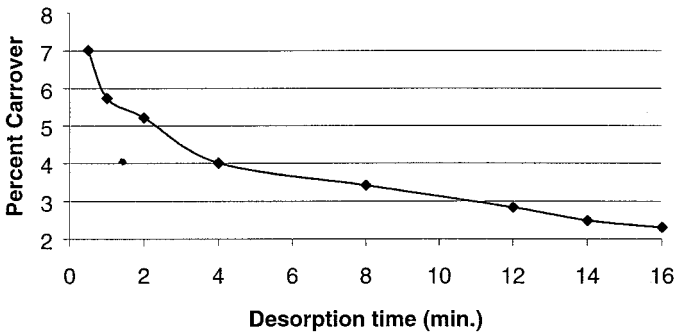

FIG. 3—*Percent carryover of GHB vs. SPME desorption time.*

Results and Discussion

Optimization of GHB Derivatization

SPME method development for the detection of GHB involved the optimization of many parameters. The derivatization of GHB was performed using a chloroformate derivatization method. Methodologies for chloroformate derivatization of carboxylic acids in aqueous solution have been developed by Hušek et al. (18–20), and chloroformate has shown to be an effective aqueous derivatization agent for several hydrophilic compounds (21,22). This aqueous method allows for the simple derivatization of GHB in the sample aliquot, after which the SPME fiber can be directly exposed to the derivatized sample. Hušek determined that only the hydroxyl groups adjacent to the carboxylic group are derivatized (18–20). Thus, in the case of GHB, the gamma hydroxyl group remains underivatized, as is seen in Fig. 1.

The efficiencies of four different chloroformate derivatization agents, ethyl-, propyl-, butyl-, and hexyl-chloroformate, were compared based on the resulting chromatographic peak areas of the four derivatized GHBs using SPME-GC/MS. In comparison, the hexyl-chloroformate adds the longest alkyl chain during derivatization, making the hexyl-derivative most amenable to GC detection. Not surprisingly, the most nonpolar chloroformate—hexyl—proved to be most effective with the largest peak area and was chosen for use throughout this study.

A base is also necessary to facilitate the derivatization reaction. The presence of base in the reaction mixture allows for the removal of the sodium counter-ion from the GHB salt, thus facilitating the reaction with the chloroformate. Three bases were compared for their reaction efficiency, pyridine, potassium carbonate, and dimethyl-aminopyridine (Fig. 2). Pyridine resulted in the largest amount of GHB detected and was selected as the base for the remainder of the study. Pyridine was also the base of choice for the Hušek studies (18–20).

Optimization of SPME-GC/MS Method in Pure Water

For the SPME method development, three different fibers, a 100 μm polydimethylsiloxane (PDMS), a 65 μm polydimethylsiloxane-divinylbenzene (PDMS-DVB), and a 65 μm carbowax-divinylbenzene (CW-DVB), were compared for extraction efficiency and carryover of GHB. Relative to the other two fibers examined, the CW-DVB fiber has a moderate extraction efficiency, but also produced the highest carryover percentage. The PDMS-DVB fiber had a high extraction efficiency, but produced the highest background in the GC/MS spectra of the three. The PDMS fiber had a relatively low extraction efficiency, but produced a low baseline and a low carryover percentage. Therefore, the 100 μm PDMS (poly-dimethylsiloxane) fiber was chosen because of its low carryover and low background, even though the extraction efficiency was lower than the other fibers analyzed.

The amount of time the SPME fiber is allowed to desorb analytes in the injection port affects the quantity of analyte that is desorbed off the SPME fiber and the amount of analyte that is retained on the fiber. Because SPME is an equilibrium process, all of the analyte will not be removed from the fiber. However, increasing the desorption time will decrease the carryover to a low equilibrium level, as is observed in Fig. 3. It was determined that after a period of 12 min in the injector port, the amount of carryover is reduced to 2 to 3% for the PDMS fiber. Thus, a 12 min desorption time allows reuse of the fiber without excessive carryover. This 12 min desorp-

tion period allows for sufficient desorption of the analyte while maintaining a relatively short analysis time.

Biological matrices contain various amounts of salt. Therefore, it is important to analyze the effect of salt concentration on SPME extraction. The presence of salt increases the polarity of the solution and changes the solvation environment of the target analytes. The increase in the ionic strength of the solution should increase the amount of analyte that is absorbed into the nonpolar SPME fiber. An enhanced SPME efficiency has been seen using this technique for the extraction of other compounds (15). The effect of adding salt to the GHB solution prior to extraction was explored. A consistent amount (2.0 mL) of various percentages of a saturated NaCl solution (25, 50, 75, and 100% saturated NaCl) were added along with 1.0 mL of a pH 7 buffer to dilute the derivatized GHB solution from 0.5 to 3.5 mL prior to extraction. Surprisingly, no significant enhancement in signal, resulting from a more efficient extraction of derivatized GHB, was observed upon detection by GC/MS. Thus, in all future experiments, salt was not added to the sample prior to extraction. The derivatized aliquot was simply diluted with deionized water and buffer solution.

GHB produces two distinct characteristic ions, 87^+ and 171^+ (Fig. 1), after derivatization and detection using GC/MS, as discussed in the experimental section. These ions are easily detected using selected ion monitoring in water samples spiked with varying amounts of GHB. Using an internal standard, GHB-d_6, a linear calibration over the concentration range of 2 to 150 μg/mL of GHB was obtained, giving a line equation of $y = 0.0752x + 0.5064$ with a correlation coefficient of 0.9954. Detection limits as low as 0.2 μg/mL were observed. These aqueous detection limits are comparable to those seen using the conventional methods (5,9,11,16). Figure 4 shows the total ion chromatogram for the detection of GHB and GHB-d_6 in a spiked water sample.

To ensure that this method selectively derivatizes and extracts only GHB relative to GBL, experiments using the same sample preparation and extraction methods described previously were carried out on an aqueous solution spiked with only GBL. The spiked aqueous solutions had a concentration 50 μg/mL of GBL, which is well above the limit of detection for GHB and within the linearity of the system. Following attempted extraction with the PDMS SPME fiber and detection using GC/MS, no peak corresponding to GBL was observed. Thus, this method is selective for GHB over GBL, and no GBL is formed prior to GC injection.

SPME of GHB Spiked Urine Samples

Although urine is a much more complex matrix, the characteristic ions for GHB were also easily detectable with selected ion monitoring for the 87^+ and 171^+ ions. However, the signals detected while using the urine matrix were lower than those seen with the water samples, presumably due to other matrix interferences found in the urine. Again using an internal standard, GHB-d_6, a linear calibration over the concentration range of 5 to 500 μg/mL of GHB was obtained, giving a line equation of $y = 0.086x + 1.2107$ with a correlation coefficient of 0.9969. Detection limits as low as 2 μg/mL were observed. The limit of detection in urine is higher than that seen for the aqueous samples due to matrix interferences in the urine. Again, this limit of detection for urinary samples is comparable to those seen using previously published methods (10,13,14).

A series of GHB/GHB-d_6 spiked urine experiments were performed to determine the accuracy of the method. Eleven spiked urine samples were prepared containing a known concentration of GHB and also GHB-d_6 for internal standard purposes. The concentrations of the samples ranged from 0 to 350 μg/mL in urine. These samples were derivatized and extracted by SPME using the previously discussed method. Each sample was run in duplicate. The results for this series of experiments are shown in Table 1. The ex-

TABLE 1—*Comparison of actual GHB spike concentration to experimentally determined spike concentration in urine.*

Sample #	Actual Concentration of Spiked GHB Sample (μg/mL)	Experimentally Determined Concentration of Spiked GHB Sample (μg/mL)
1	300	334
2	50	73
3	350	361
4	225	212
5	90	125
6	25	35
7	250	265
8	100	143
9	335	347
10	150	139
11	…	none detected

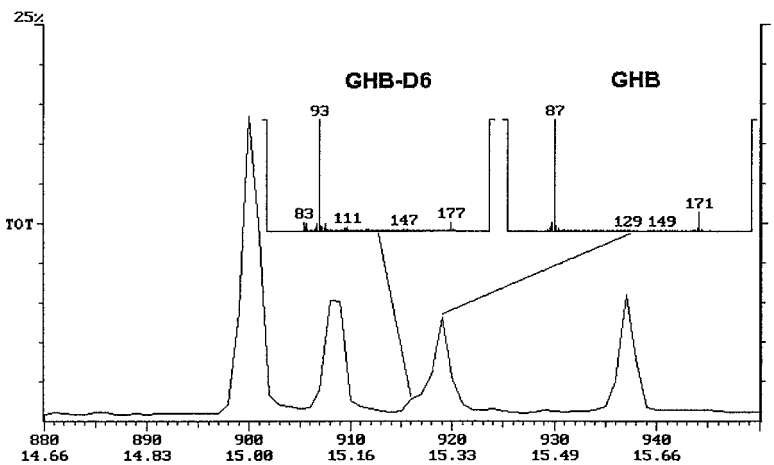

FIG. 4—*SPME-GC/MS spectrum of GHB and GHB-D_6 in a spiked water sample.*

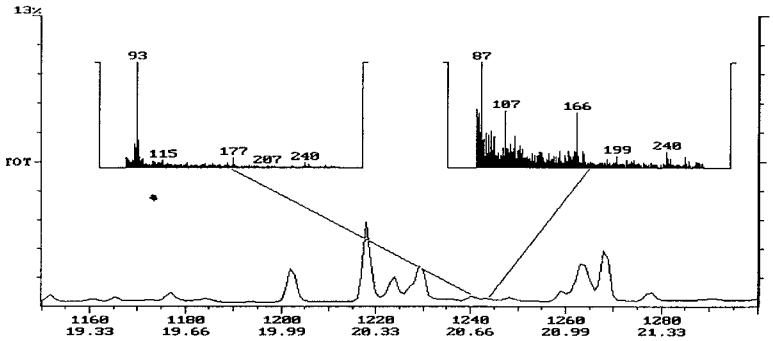

FIG. 5—*SPME-GC/MS spectrum of GHB and GHB-D_6 in a "blank" urine sample.*

TABLE 2—*Experimentally determined GHB concentration of case urine sample by the hexyl chloroformate/SPME method and conventional analysis.*

Case #	SPME Determined GHB Concentration (μg/mL)	Conventionally Determined GHB Concentration (μg/mL)
1	130	148
2	4730	5140
3	406	415
4	951	716
5	5400	5316

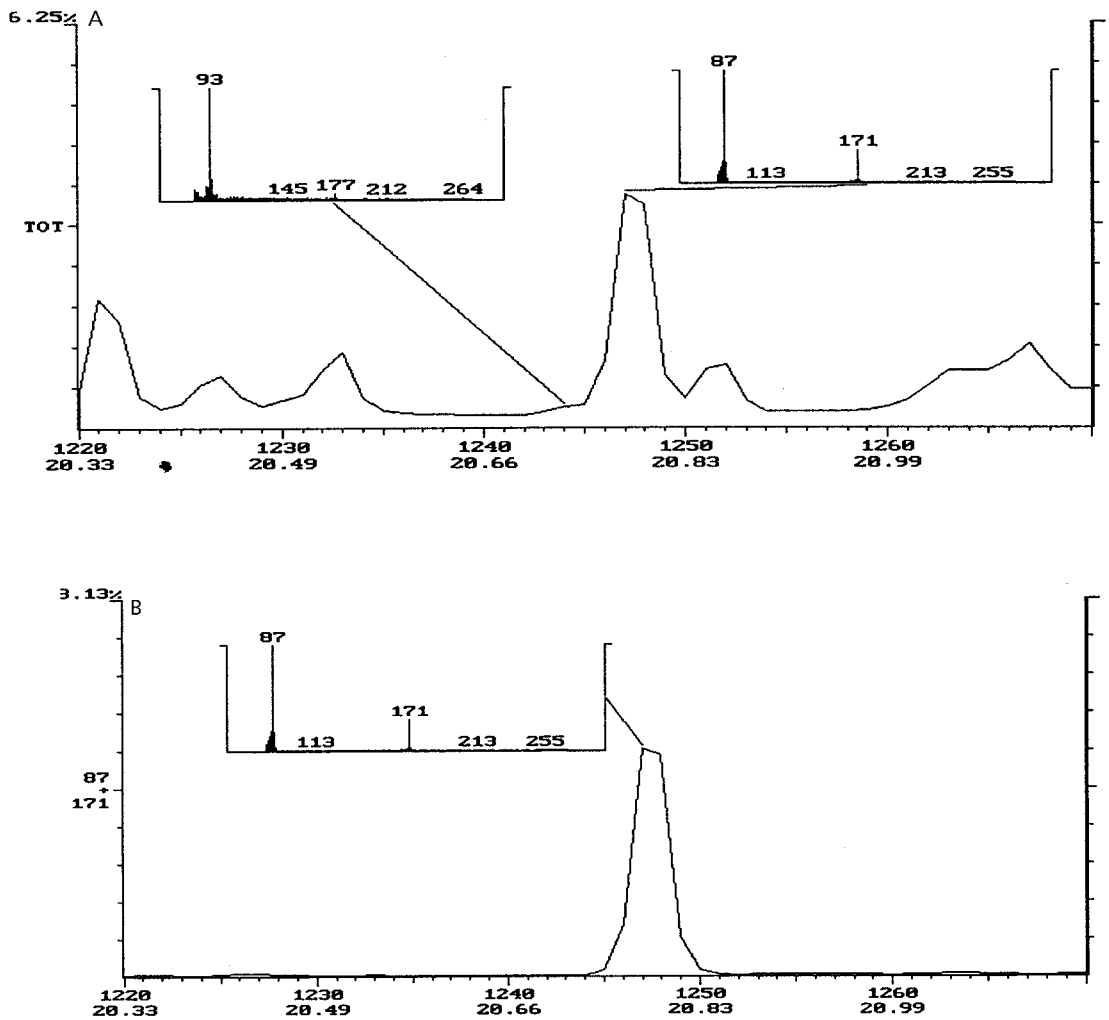

FIG. 6—*SPME-GC/MS spectrum of GHB in a case urine sample.* (A) *Total ion chromatogram.* (B) *Selected ion monitoring for 87^+ and 171^+.*

perimental results show good correlation with the known spike amounts and have an average error of approximately seventeen percent. The values are more precise for the higher concentrations than the lower spike concentrations. The standard deviation of inter- and intra-day samples remained less than 10% for both the spiked aqueous and spiked urine samples due to the use of an internal standard. The accuracy of this method is comparable to results seen using a more conventional method (14). The chromatogram of the extract from a "blank" urine sample, specifically Sample 11 in Table 1, is shown in Fig. 5. Trace amounts of GHB and GHB-d_6 can be seen from carryover on the SPME fiber.

Clinical Urine Specimens

Four clinical urine specimens suspected to contain GHB and one urine specimen from an accidental GHB overdose were analyzed for GHB by both the hexyl chloroformate/SPME-GC/MS method ($n = 3$) and a conventional GC/MS method ($n = 2$). The results are summarized in Table 2. Figure 6*A* shows the total ion chromatogram for the SPME detection of GHB in the overdose urine sample. Likewise, Fig. 6*B* displays the chromatogram using selected ion monitoring for the 87^+ and 171^+ mass spectral peaks. The results from both methods agree well, thus indicating good accuracy is attained by both methods. A significant difference between the two methods is the use of a deuterated internal standard for GHB throughout the entire process for the hexyl chloroformate/SPME method. In contrast, the conventional method utilizes only an internal standard for the extraction portion of the method. As mentioned previously, the hexyl chloroformate/SPME method measures only GHB and does not rely on the conversion of GHB to GBL. In the GC/MS conventional method, a separate analysis of the specimen without conversion of GHB to GBL to correct for native GBL in the specimen is required.

Conclusions

In this study, SPME-GC/MS has been applied to the determination of gamma-hydroxybutyrate. SPME-GC/MS is a valuable qualitative and quantitative tool for the detection of GHB, providing a simple, rapid method. The hexyl-chloroformate derivatization reaction is the key to allowing SPME-GC/MS monitoring of GHB directly in water and urine samples. This method is quantitatively linear over several orders of magnitude with the addition of an internal standard, GHB-d_6. Limits of detection as low as 0.2 μg/mL in spiked aqueous samples and 2 μg/mL in spiked urine samples have been reached. Results obtained for sensitivity and linearity are comparable to those seen using traditional methods of separation, yet the SPME method is superior due to the simplicity, speed of analysis, reduction in solvent waste, and ability to differentiate between GHB and GBL. Further studies are warranted to test the reliability of a conventional liquid-liquid or solid phase extraction for the isolation of the hexyl derivative of GHB for laboratories which do not have access to SPME technology.

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