GHB Free Acid: II. Isolation and Spectroscopic Characterization for Forensic Analysis

ABSTRACT: A reference standard for γ-hydroxybutyric acid (GHB) free acid is not commercially available, making its analysis in forensic exhibits more difficult. GHB free acid is typically encountered in aqueous solution and in the presence of the lactone, γ-butyrolactone (GBL), presenting difficulty in Fourier transform infrared (FT-IR) analysis. The strong infrared (IR) absorptivity of the GBL carbonyl band, the shifting of the GBL carbonyl band in aqueous solutions, and the position of the O–H bend for water can mask the main carbonyl band for GHB free acid. Neither of these chemical handbooks provides the primary reference material, small amounts (≤10 mg) of GHB free acid. Preparation was based on the instantaneous reaction of GHB’s sodium salt with a stoichiometric amount of hydrochloric acid in aqueous solution, and subsequent isolation of the free acid in neat liquid form. Both FT-IR and proton nuclear magnetic resonance spectra of the neat reference material were obtained and used to verify its identity. The isolation of GHB free acid from actual forensic exhibits is also presented, with identity confirmation using FT-IR.

KEYWORDS: forensic science, GHB free acid, γ-hydroxybutyric acid, GHB, gamma-hydroxybutyrate, spectroscopic analysis, isolation, FTIR, NMR, 1H NMR, GBL, interconversion, sodium oxybute

Although it is well known that γ-hydroxybutyric acid (GHB) free acid exists in aqueous acidic solutions of GBL, methods for large-scale isolation of the free acid from the lactone have not been reported. In the previous paper (1), we examined the difficulties associated with the analytical detection of GHB free acid in aqueous-based forensic samples, including the discrimination between GHB free acid and salt forms. This discrimination is made more difficult because analytical standards or reference materials for GHB free acid are not commercially available. In this work, the particular difficulties associated with the IR analysis of GHB free acid in aqueous solutions were studied, both for understanding and to develop appropriate methods. Aqueous solutions of β-hydroxybutyric acid (BHB) and GBL were studied in order to further understand the masking of the GHB free acid carbonyl band in FT-IR analysis. The use of second derivative FT-IR spectroscopy was shown to provide resolution of the free acid carbonyl band, and a presumptive test for GHB free acid was developed and applied. An extension of this work included preparing, for use as a standard reference material, small amounts (≤10 mg) of GHB free acid. Preparation was based on the instantaneous reaction of GHB’s sodium salt with a stoichiometric amount of hydrochloric acid in aqueous solution, and subsequent isolation of the free acid in neat liquid form. Both FT-IR and proton nuclear magnetic resonance spectra of the neat reference material were obtained and used to verify its identity. The isolation of GHB free acid from actual forensic exhibits is also presented, with identity confirmation using FT-IR.

We report an approach to rapidly prepare and isolate small amounts (≤10 mg quantities) of GHB free acid for use as a reference material. We investigated the instantaneous production of GHB free acid in solution from reaction of the sodium salt with a stoichiometric amount of hydrochloric acid, and subsequent isolation in its neat form via rapid evaporation and washing of the residue. Both IR and proton nuclear magnetic resonance (1H NMR) spectroscopy were used to verify the identity of the reference material, to discriminate between GHB free acid and salt, and to check for formation of GBL. High-performance liquid chromatography with ultraviolet detection (HPLC-UV) was used to determine the yields of free acid produced from the salt and to monitor for the presence of GBL.

Interconversion, reactivity, and analytical issues for GHB in forensic evidence continue to represent areas of interest and study (8–10), including an approach based on sampling via solid phase microextraction, followed by on-fiber derivatization and GC-MS analysis (10). In our work, after preparation of the GHB free acid reference material, the isolation and detection of the free acid in actual forensic samples was investigated. Unlike the previously reported approach for forensic analysis of GHB free acid (7), our purpose was not to intentionally convert GHB to the free acid for forensic identification. Rather, our goal was to detect and identify any GHB free acid already present in forensic evidence at the time of analysis and to avoid altering the relative proportions of GHB free acid, GHB carboxylate, and GBL in a given sample. For this reason, no adjustments in solution pH were made before free acid isolation from actual forensic samples.

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Materials and Methods

Standards and Chemicals

GHB acid, sodium salt (NaGHB, minimum 99%) was obtained from Sigma (St. Louis, MO). BHB acid (BHB, 95%), and BHB acid, sodium salt (NaBHB, 99%) were obtained from Aldrich (Milwaukee, WI). γ-butyrolactone (GBL, 99+% was obtained from Aldrich or Sigma. Hydrochloric acid (Baker Analyzed) was obtained from JT Baker (Phillipsburg, NJ). Deionized water was obtained from a Millipore Milli-Q filtration system, or EM Science (Omnisolv water, Gibbstown, NJ).

BHB Acid Modeling Experiments

A series of solutions was prepared using BHB, GBL, and water. The concentrations and ratios of BHB and GBL were prepared to mimic the concentrations of GHB free acid and GBL observed in aqueous-based GBL formulations encountered by the FDA and DEA laboratories, and ranged from 20 to 75 mg/mL for BHB free acid and 67–385 mg/mL for GBL (see Table 1). Single component solutions of either BHB free acid or GBL were prepared at the same concentrations as the mixed solutions for direct comparison. The samples were analyzed immediately by FT-IR (FT-IR measurements in duplicate) and HPLC-UV.

Free Acid Reference Material Isolation Experiments

For free acid isolation, small volume aliquots (3–25 μL) of the aqueous NaGHB/HCl reaction mixtures (1) were drawn for deposition onto clean BaF2 windows or into clean glass Petri dishes. The water was evaporated using the heat generated from an ordinary desk lamp with a standard 100 W soft white bulb placed approximately 6 in. above the BaF2 window or Petri dish. Drying times ranged from 5 to 15 min depending on the deposition volume. A few experiments were also conducted in which the deposited aliquots were dried in a desiccator under vacuum.

For the stoichiometric reaction mixtures, the dried residue consisted of GHB free acid and sodium chloride. The dried residue was subsequently extracted with a small volume of chloroform in order to liberate the GHB free acid from the NaCl crystals. The liberated free acid consisted of a ring of small liquid droplets that formed concentrically around the original deposition site. IR measurements (microscope) were obtained for the residue dried on the BaF2 window before the chloroform wash step, and for the liquid droplets formed after the chloroform extraction.

The nonstoichiometric reaction mixtures contained either excess GHB salt or GBL (formed from dehydration in the presence of D2O) or into clean glass Petri dishes. Drying was performed using the heat generated from an ordinary desk lamp with a standard 100 W soft white bulb placed approximately 6 in. above the BaF2 window or Petri dish. Drying times ranged from 5 to 15 min depending on the deposition volume. A few experiments were also conducted in which the deposited aliquots were dried in a desiccator under vacuum.

The nonstoichiometric reaction mixtures contained either excess GHB salt or GBL (formed from dehydration in the presence of D2O), depending on the reagent ratios. It was noted that GBL tended to evaporate efficiently under these drying conditions. In the case of the excess GHB salt experiments (acid limited), a second concentric ring of residue was observed close to the original deposition site. IR measurements were also taken of this residue, which consisted of the GHB carboxylate.

1H NMR Measurements

For 1H NMR measurement, the dried residues were reconstituted in D2O and analyzed using the same conditions as previously reported (1). The 1H NMR data, comprising of two triplets (3.615 and 3.42 ppm) and one quintet (1.827 ppm), indicated the presence of only the GHB free acid. In fact, over a 12 h period, no GBL formation was detected in this D2O solution.

IR Spectroscopic Measurements

IR measurements of the dried residues (see “Free Acid Reference Material Isolation Experiments”) were obtained using the microscope sampling accessory from a Nicolet Magna 550 FT-IR and Perkin-Elmer Spectrum GX FT-IR with an AutoImage System Microscope. For all the IR microscopy measurements, a diamond anvil cell (Diamond Optics) was used in the open-faced configuration. A small portion of the liquid sample was mounted on the diamond anvil cell, and a background was collected from a clean portion of the diamond anvil cell.

IR measurements for aqueous-based GBL products and spiked beverages were obtained using a Nicolet Magna 550 FT-IR with a SensIR Durascope (Smiths Detection, Danbury, CT) single reflectance attenuated total reflectance (ATR) accessory. The internal reflectance element (IRE) of the ATR accessory was composed of zinc selenide (ZnSe) coated with diamond. For measurement of the neat liquid, a 10 μL aliquot of sample was spotted directly onto the ATR window. For isolation and identification of GHB free acid from forensic samples, a 25 μL aliquot of sample was spotted onto a tin oxide coated glass microscope slide (SensIR Low E slide). The slide was placed in a 110°C oven for approximately 15 min, removed and allowed to cool. The slide was then inverted and the deposition site placed over the ATR window for IR measurement of the dried residue. A small amount of pressure (not registered by load sensor) was applied to the slide to hold its position.

Heated stage experiments were conducted using a Travel IR FT-IR spectrometer (SensIR) with a nine reflection ATR accessory and a stage temperature of 70°C (nominal). An aliquot (10–100 μL) of sample was spotted directly onto the ATR window. The sample was allowed to dry and then an IR spectrum collected.

All ATR sample spectra were ratioed against a clean ATR IRE background (air). All data were ATR corrected using the ATR correction in the Nicolet Omnic software version 6.0. All IR microscopy sample spectra were ratioed against a clean background spectrum obtained from a clean portion of the open-faced diamond anvil cell. The data collection parameters for each of the instruments are given in Table 2. In the case of the GBL:BHB mixture spectra and sample spectra, the corrected ATR spectra were further processed using a second derivative function (Galactic Grams software version 5.24). A Savisky–Golay second derivative function (3 point polynomial with a 9 point smooth) was used to process the data.

HPLC-UV Quantitative Analysis of GHB, BHB, and GBL

See Part 1 (1) for details of the HPLC systems and conditions. The retention times for GHB, BHB, and GBL were 5.9, 7.1, and 8.9 min.
TABLE 2—Fourier transform infrared (FT-IR) spectrometer data collection parameters.

| Parameter          | Nicolet Magna with Microscope | Nicolet Magna Durascope ATR | Perkin Elmer Microscope |
|--------------------|--------------------------------|----------------------------|-------------------------|
| Attachment         | None                           | ATR                        | None                    |
| Resolution         | 4 cm⁻¹                         | 4 cm⁻¹                     | 4 cm⁻¹                  |
| Number of coadditions | 128                           | 64                         | 128                     |
| Spectral range     | 4000–750 cm⁻¹                  | 4000–650 cm⁻¹               | 4000–750 cm⁻¹            |
| Apodization function| Happ-Genzel                    | Happ-Genzel                | Strong                  |
| Gain               | 2                              | 1                          | 2                       |
| Detector           | MCT                            | DTGS                       | MCT                     |

ATR, attenuated total reflectance.

7.6 min, respectively. Standard solutions for GHB and BHB were each prepared from the respective sodium salt. For HPLC analysis, the dried residues were reconstituted in 1.0 mL water.

Results and Discussion

Forensic evidence encountered at the FDA laboratory in the form of aqueous-based GBL solutions has typically contained GBL in the 2–15% w/v range with solution pH in the 2–5 range (11). As solutions in the pH 2–3 range had already reached equilibrium concentrations of GBL and GHB free acid, no further changes in solution pH were observed over time (some products monitored over a 4-year period). For solutions in the pH range 3–5, a net increase in the concentration of GHB free acid continued until equilibrium was reached and the pH stabilized in the 2–3 range (11). Under equilibrium conditions, the molar ratio of GHB free acid to GBL was ca. 1:2 (11). Based on the solution pH at equilibrium (pH 2–3), the predominant form of GHB was the free acid.

In Part I (1), the acid–base aqueous solution chemistry of GHB was examined, particularly with respect to formation of GHB free acid and spectroscopic detection of GHB free acid in solution. ¹HNMR provided a convenient and ideal means for direct analysis of forensic evidence comprised of aqueous solutions containing GHB free acid, GHB carboxylate, and GBL. ¹HNMR proved effective at discriminating between the GHB free acid and carboxylate forms. Despite the obvious value of NMR for this type of analysis, NMR instrumentation is rarely found in the forensic laboratory because of budgetary reasons.

Fortunately, FT-IR is a powerful identification technique that can discriminate between the carboxylic acid free acid moiety and the deprotonated carboxylate. Unfortunately, direct IR analysis of the neat sample liquid for aqueous-based sample evidence containing GHB free acid and GBL is complicated by interference from the water band at 1650 cm⁻¹ (OH bend). In this part of the study, we examined the prominent IR spectral features of GHB (both free acid and carboxylate forms) and GBL, and used a second derivative spectral data processing technique to “resolve” these spectral features from the water (OH bend) interference in the spectra of the neat liquid solutions. We then developed a procedure for physical isolation of GHB free acid to generate both a reference material for addition to our in-house IR spectral library, which was used in the analysis of GHB in forensic evidence using FT-IR spectroscopy.

IR Spectra of Aqueous-Based GBL Solutions and Related Compounds

NaGHB and GBL neat liquid are readily distinguishable by their IR spectra. The most prominent bands are the carboxylate [O= - C= - O] “bond and a half” asymmetric stretch for NaGHB at 1558 cm⁻¹ and the C=O carbonyl absorbance for GBL at 1762 cm⁻¹ (12–14). Likewise, aqueous solutions of GBL and the sodium salt of GHB are readily distinguishable (Fig. 1), despite the presence of the O–H bend of water at 1640 cm⁻¹. When a GBL solution is freshly prepared, there is no measurable GHB free acid present in solution. However, a shift in the band position of the lactone carbonyl to 1743 cm⁻¹ occurs relative to that of the pure compound at 1762 cm⁻¹ because of hydrogen bonding with water (15).

The IR spectrum for a typical aqueous-based GBL product (neat liquid sample, no sample dilution) is given in Fig. 2, with the region from 2,100–1,500 cm⁻¹ inset and expanded for clarity. Through separate analysis using HPLC-UV, the concentrations of GBL and GHB (calculated as the free acid) in the GBL product were determined to be 8.7% w/v and 4.6% w/v, respectively, at a product pH of 2.8 (neat liquid). Despite the presence of the free acid in the product at nearly 5% w/v, no band from the free acid carbonyl was readily distinguishable. Rather, two prominent broad bands are observed at 1650 and 1743 cm⁻¹ corresponding to water (OH bend) and GBL (carbonyl), respectively (Fig. 2, expanded inset).

The reasons for the masking of the free acid carbonyl band can be explained as follows. The carbonyl stretch of an aliphatic carboxylic acid such as GHB free acid is normally observed in the range 1720–1710 cm⁻¹ for the pure compound. However, as was the case with the lactone, the carboxylic acid carbonyl band shifts slightly to a lower wavenumber position in aqueous solution because of hydrogen bonding. The degree of shift in the carboxylic acid carbonyl is much less than that observed for the lactone carbonyl, resulting in a moderately sharp band near 1710 cm⁻¹ for the free acid in aqueous solution. However, the free acid carbonyl band is masked by the presence of the two other broader bands: the OH bend of water at 1650 cm⁻¹, and the strong absorbance band for the GBL carbonyl at 1743 cm⁻¹, occurring in the “envelope” region beneath this set of overlapping bands (Fig. 3). Although IR spectroscopy has been a technique of choice to identify various GHB salt forms and GBL in forensic evidence with little to no sample preparation, the presence of the free acid is not readily detected in aqueous-based GBL formulations. Rather, the IR spectra of these formulations resemble those of freshly pre-
pared GBL solutions (see also Fig. 3), which have no measurable free acid present.

BHB Modeling Experiments and Presumptive Screen for GHB Free Acid: Second Derivative IR Spectroscopy

Several experiments were conducted to further understand the IR band envelope observed in the carbonyl region (1750–1650 cm\(^{-1}\)) for GBL:GHB free acid aqueous solution samples. As GHB free acid is not commercially available, BHB was substituted as a model compound. BHB represents a valid model for GHB free acid because the position of the carbonyl band (C = O) for BHB is the same as that for GHB free acid (1,710 cm\(^{-1}\)). Minor differences in other regions of the IR spectra of GHB and BHB, ignored for this series of experiments, were because of the location of hydroxyl group (OH) in BHB on the butyl backbone and the presence of a terminal methyl group (CH\(_3\)), whereas GHB contains only methylene groups (CH\(_2\)).

Preparation of GBL:BHB solutions enabled us to replicate and evaluate the band envelope observed with GBL:GHB free acid aqueous solutions. The concentrations and ratios of BHB and GBL were prepared to mimic the concentrations of GHB free acid.

![FIG. 2](image1.png)---Fourier transform infrared spectrum of a typical aqueous-based \(\beta\)-butyrolactone (GBL) product that contains ca. 5% w/v \(\gamma\)-hydroxybutyric free acid. Inset: expanded view showing the predominant GBL and water bands.

![FIG. 3](image2.png)---Expanded region Fourier transform infrared spectrum (1900–1470 cm\(^{-1}\)) for an aqueous-based \(\beta\)-butyrolactone (GBL) product containing \(\gamma\)-hydroxybutyric (GHB) free acid, compared with aqueous solutions containing only GBL or GHB free acid, and compared to water. The position of the free acid carbonyl is denoted by the vertical line.
and GBL previously encountered in aqueous-based GBL forensic evidence at the FDA and DEA laboratories (see Table 1, solution nos. 1C, 2C, and 3C). Note that the GBL concentrations are typically greater than 2:1 relative to the GHB free acid concentrations prior to equilibrium, and approximately 2:1 at equilibrium. Single component solutions of either BHB free acid (solution nos. 1B, 2B, and 3B in Table 1) or GBL (solution nos. 1A, 2A, and 3A in Table 1) were prepared at similar concentrations as the mixed solutions for direct comparison. The overall range of concentrations tested was 20–75 mg/mL for BHB and 67–385 mg/mL for GBL.

Initially, the IR spectra from separate solutions of GBL (Fig. 4) and BHB (Fig. 5) were examined. The carbonyl band (C=O) associated with GBL and the OH bend of water were not baseline resolved, although significant separation between the peaks was observed (Fig. 4A). For the BHB solutions, the carbonyl band (C=O) appeared as a shoulder to the OH bend of water (Fig. 5A). Given the band position for the BHB carbonyl, the overlap of the GBL carbonyl band and the OH bend of water would be expected to hinder identification of the GHB free acid carbonyl in aqueous GBL:GHB solutions based on simple inspection of the IR spectrum. Thus, we investigated the use of post data collection data treatment, using a second derivative function to resolve the band overlap.

A second derivative function was applied to the spectra of the individual GBL and BHB solutions. For the GBL solution, two
distinct peaks were observed in the second derivative spectrum representing the GBL carbonyl and the OH water bend (Fig. 4B). For the BHB solution, the BHB carbonyl was resolved from the OH water bend in the second derivative spectrum (Fig. 5B). The respective peak intensities in the second derivative spectra increased with increasing concentrations of GBL and BHB (Figs. 4B and 5B).

Next, mixed aqueous solutions of GBL and BHB were analyzed, and their second derivative data evaluated to determine if all three species (BHB, GBL, and water) could be observed (Fig. 6). Figure 6B is the second derivative spectrum for a mixture of BHB (conc) relative to GBL (conc) in water. In this spectrum, three distinct second derivative bands were observed corresponding to the GBL carbonyl (position), BHB carbonyl (position), and water OH bend. Resolution of the three peaks/bands was observed over the range of concentrations tested (Fig. 6B).

Finally, a series of aqueous-based forensic samples, which had been previously analyzed for GHB and GBL using GC-MS, HPLC-UV, and absorbance FT-IR, were selected for further analysis using FT-IR with the second derivative function. In addition to GBL and GHB, these samples were known to contain sugar, citric acid, low levels of flavors, and a dye. The samples were also reanalyzed for GHB and GBL content (HPLC) to check for changes as the original analysis; no changes were noted indicating equilibrium concentrations of GHB and GBL had previously been established. In all cases, distinct peaks corresponding to the GBL carbonyl (1746 cm⁻¹), GHB free acid carbonyl (1710 cm⁻¹), and the OH bend of water (1650 cm⁻¹) were observed (Fig. 7, spectrum shown for one forensic sample), corresponding to the same band positions as previously observed for the GBL:BHB model mixtures. The second derivative spectrum for a freshly prepared GBL solution (contains no measurable free acid), and a freshly prepared GHB free acid solution are also given for comparison (see Fig. 7).

These results demonstrate that second derivative FT-IR spectroscopy can provide a presumptive screen for GHB free acid via direct analysis of aqueous-based GBL:GHB forensic evidence. This approach provided the major advantage that essentially no sample manipulation was required prior to analysis. These results also confirm the difficulty of detecting GHB free acid using FT-IR absorbance measurements of neat liquid samples. For definitive confirmation using FT-IR, we subsequently investigated isolation of the free acid.

### Isolation of GHB Free Acid Reference Material

In Part I (1), aqueous stoichiometric mixtures of NaGHB and HCl were prepared in order to generate the GHB free acid in solution which was then detected by 1HNMR and FTIR. Reaction of NaGHB with HCl produced the free acid almost instantaneously. Immediately following the formation of the free acid, the solution pH quickly stabilized in the range pH 2.2–2.6 through dissociation of the newly protonated GHB free acid. In this pH range, a minute amount of the carboxylate also reforms because of acid dissociation (< 1 mol%). These stoichiometric solutions were comprised primarily of GHB free acid, sodium chloride, and water. For isolation of the free acid in this second study, the goal was to remove water as quickly as possible without promoting formation of lactone, and then to isolate the free acid neat liquid from the sodium chloride through solvent extraction. All reaction mixtures were prepared immediately prior to the deposition and isolation experiments. 1HNMR measurements made on the stoichiometric reaction mixtures showed these to be stable for a period of at least 12 h, without any detectable amount of lactone formed.

In the initial experiments, 3 μL aliquots of the reaction mixtures were deposited onto a clean barium fluoride (BaF₂) window, and the water was evaporated using the heat from an ordinary desk lamp (see Experimental section for further details). This cautious
approach to removal of water seemed prudent given the limited physical data for the GHB free acid available in reference sources (1). More aggressive heating may have caused evaporation and/or decomposition.

When observed under a stereolight microscope, the residue appeared as a dried mass. This mass was saturated with a minimal volume of chloroform (ca. 25 \( \mu \)L); the chloroform was allowed to evaporate, which resulted in the formation of oil-like liquid droplets in a concentric ring around the original point of deposition. The sodium chloride residue remained at the point of deposition. The IR spectrum of the droplets obtained using an IR microscope revealed the presence of pure liquid GHB free acid (Fig. 8). The IR absorbance bands observed in the IR spectrum in Fig. 8 are consistent with the molecular structure of GHB free acid. Table 3 provides the peak positions and band assignments for the individual peaks observed in Fig. 8. Several additional independent preparations of the free acid using this technique confirmed these results. To our knowledge, there has been only one other published IR spectrum for GHB free acid (7) and our results are consistent with the published work.

It was necessary to tightly control the amount of HCl added to the reaction mixture in order to avoid formation of the lactone or incomplete conversion of the salt to the free acid. Figures 9A–C show the mid-region IR spectra of the liberated droplets (dried residue after chloroform extraction) for the stoichiometric reaction mixture (Fig. 9B), compared with that from reaction mixtures with a slight acid-limited mole ratio (HCl mole amount ca. 9% less than stoichiometric amount, Fig. 9A), and a slight acid-excess mole ratio (HCl mole amount ca. 4% greater than stoichiometric amount, Fig. 9C). Evidence of residual GHB salt (absorbance at 1558 cm\(^{-1}\)) was observed in the spectrum of the acid-limited reaction, whereas lactone formation (lactone carbonyl band emerging, GBL) was observed in the spectrum of the acid-excess reaction. The use of the lamp in the initial drying step (water removal) provided for less rigorous heating conditions relative to either a hot plate or oven. The relatively mild heating conditions chosen in these experiments allowed for observation of the lactone (Fig. 9C), which may have been completely volatized under more aggressive conditions. See also the discussion under “Potential for Interconversion: Heated Stage and Spiking Experiments,” in which the observed evaporation order was water first, followed by the lactone, and lastly, the free acid.

| Band | Peak Position (cm\(^{-1}\)) | Band Assignment/Functional Group |
|------|-----------------------------|---------------------------------|
| 1    | 3274                        | OH stretch associated with the OH group in the \( \gamma \) position |
| 2    | 2958                        | C–H asymmetric stretch associated with CH\(_2\) groups |
| 3    | 2893                        | C–H symmetric stretch associated with CH\(_3\) groups |
| 4    | 2633                        | O–H stretch associated with the OH group of the carboxylic acid |
| 5    | 1712                        | Carboxylic acid carbonyl stretch |
| 6    | 1416                        | Interaction of C–O stretch and C–O–H in plane bend associated with the OH group in the \( \gamma \) position |
| 7    | 1057                        | C–C–O stretch associated with the OH group in the \( \gamma \) position |

GHB, \( \gamma \)-hydroxybutyric acid; IR, infrared.

**TABLE 3**—Peak positions and IR band assignments for GHB free acid.
In subsequent experiments, larger volumes of the reaction mixtures (10–100 μL) were taken for deposition and free acid isolation. Larger volume depositions required extended drying periods (up to 20 min). Reaction yields for preparation of the free acid were determined by reconstituting the residues in a suitable volume of water, followed by HPLC analysis. Analysis of these residues was conducted at two points: first, after deposition/drying and second, after chloroform extraction/evaporation. Reaction yields for the stoichiometric reaction mixtures were comparable for reconstitution of the residue at the two points in the procedure (89–97% for deposition/drying and 89–92% for chloroform washing/evaporation). Yields were generally lower from the larger volume depositions, probably because of the longer drying time required. The presence of even a slight excess of acid caused significant decreases in yields of GHB free acid (46% yield with a ca. 13% molar excess), with concomitant GBL formation. The loss in yield was likely because of GBL formation via acid catalysis followed by evaporation of some portion of the GBL (i.e., depletion of GBL causes a shift in equilibrium away from free acid). The GBL formed in acid excess experiments was observed both in the IR spectra (as shown in Fig. 9C for a 4% stoichiometric HCl excess), and in the HPLC experiments. For use as a reference material, GHB free acid was always prepared at the time of use.

**Measurement of GHB Free Acid in Forensic Samples**

For IR measurement, it was already demonstrated that the spectrum of GHB free acid is masked in measurements made on neat liquid aqueous-based GBL samples (vide infra), whereas ¹H NMR provides a convenient means for direct analysis of these sample types. However, we discovered that the general evaporation approach used for isolation of the GHB free acid from the NaGHB/HCl reaction mixtures could also be applied to the isolation of the free acid from forensic samples, followed by IR identification of the free acid.

Aliquots of aqueous-based GBL products were spotted onto low actinic glass slides, and then dried in an oven (110–115°C) for 15 min. After cooling, the IR spectra of the residues were obtained by inverting the glass slide over the window of an ATR accessory. Depending on the presence of other matrix components, the resultant IR data represented either a very clean spectrum of the free acid (Fig. 11B) or a mixture of the free acid and other nonvolatile matrix components such as carbohydrates (Fig. 11A) with the primary band for the free acid carboxyl (ca. 1710 cm⁻¹) observed in both samples.

**¹H NMR Confirmation of the Free Acid Reference Material**

In the previous paper (1), ¹H NMR was used to discriminate between the GHB free acid and salt forms based on the change in chemical shift, which occurs for the three sets of protons in the methylene chain. Likewise, ¹H NMR was used to confirm the identity of the free acid reference material in this work (Fig. 10). As seen previously (1), the chemical shifts for all three sets of protons are shifted downfield for the free acid relative to the salt form, with the largest downfield shift observed for the methylene protons adjacent to the carboxylic acid moiety. GC-MS was not used for confirmation as it does not discriminate between the GHB free acid and carboxylate forms. We also investigated the use of direct infusion electrospray MS in both APCI and negative ion ESI modes as a possible means of confirmation/discrimination. The freshly prepared free acid was reconstituted in methanol and compared with a methanolic solution of the sodium salt. However, results for the free acid and salt forms were ambiguous, with similar spectra obtained for both forms in both detection modes.
Potential for Interconversion: Heated Stage and Spiking Experiments

In hydrolysis studies, the rate of conversion of GBL to GHB is known to increase upon heating (11,16,17). The boiling point of water at 760 mmHg is 100°C and for GBL is 204–206°C (3,18). GHB free acid reportedly decomposes at 178–180°C (3), and therefore, does not have a defined boiling point. Given the minimal volumes and the range of evaporation conditions used in this study, we speculated that for stoichiometric reaction mixtures, evaporation may occur prior to any significant interconversion. Additional experiments were conducted to further investigate the relative evaporation rates for GHB free acid, GBL, and water, and to address the potential for GHB/GBL interconversion during the evaporation process.

A series of experiments was conducted in which solution aliquots were deposited onto an ATR window mounted on a heated stage (nominal stage temperature 70°C) and IR spectra of the sample were acquired of the samples throughout the evaporation process. Evaporation experiments were conducted using 10 µL aliquots of unbuffered (nominal pH 7) and buffered (pH 1.4 and 2.0, phosphate buffer) aqueous solutions of GBL, both freshly prepared at nominally 64 mg/mL. Under these conditions, evaporation was complete in ca. 4–6 min. Examination of the IR spectra throughout the evaporation process showed the disappearance of the OH bend of water (1,640 cm⁻¹), followed by disappearance of the GBL carbonyl band (1743 cm⁻¹). Both water and GBL evaporated to completion with no evidence of GHB free acid formation.

Heated stage evaporation experiments were subsequently conducted with a commercial aqueous-based GBL product that had previously reached equilibrium with interconversion of ca. one third of the GBL to free acid GHB. The GBL content was 60 mg/mL, and the GHB content was 33 mg/mL (determined by HPLC), with a solution pH of 2.8. Aliquots of 100, 25, and 10 µL were tested. In all cases, the water band was observed to diminish, followed by the GBL band, with subsequent emergence of the GHB free acid band. Evaporation rates varied with aliquot volume: evaporation of GBL was complete within 3 min for the 10 and 25 µL aliquots, but still incomplete after 30 min for the 100 µL aliquot.

Finally, spiking experiments were conducted in which GBL and GHB free acid were spiked into separate preparations of an acidic aqueous product matrix (pH 2.6). This product matrix was an actual forensic sample that contained citric acid, sugar, dye, and an aqueous product matrix (pH 2.6). This product matrix was an actual forensic sample that contained citric acid, sugar, dye, and only traces amounts of GBL (0.2 mg/mL) and GHB (0.1 mg/mL) before spiking. GBL was spiked into the product matrix at 110 mg/mL. In separate experiments, NaGHB was spiked into the product matrix at 4.4 and 25 mg/mL, and the solution pH was adjusted to 2.5–2.8 for conversion to the free acid. Sample aliquots (25 µL) were subsequently deposited onto low actinic glass slides, and evaporation was conducted in a laboratory oven at 110–115°C.

The IR spectra for dried residues from the unspiked product matrix, the product matrix spiked with GBL, and the product matrix spiked with GHB are given in Fig. 12. A small band corresponding to the GHB free acid carbonyl (1710 cm⁻¹) was observed for both the unspiked product matrix and the GBL-spiked matrix. However, the size of the free acid carbonyl band did not increase despite the high GBL concentration (110 mg/mL). Although minor band intensity differences were observed, the intensities of all peaks increased proportionally indicating that the difference in intensity between the unspiked and GBL-spiked residues was the result of the ATR sampling and not an actual increase in the amount of GHB present in the sample. No band was observed for the lactone carbonyl in the GBL-spiked matrix. These results are consistent with complete evaporation of GBL, and no interconversion of GBL to GHB.

Moreover, the band for the GHB free acid carbonyl increased for the GHB-spiked matrices as the spike amount of GHB was increased, with no evidence of GBL in the spectrum. A minor shift in the peak position was observed because of matrix and concentration effects. A small amount of residual carboxylate was also observed in the spectrum for the high GHB spike (25 mg/mL, Fig. 12), indicating incomplete conversion to the free acid. The spectrum of the dried residue for another actual forensic exhibit with a similar product matrix was also obtained after oven drying. A large free acid carbonyl band was also observed for this sample, with no GBL carbonyl band in the spectrum (GBL content of the liquid was 97 mg/mL; GHB content of the liquid was 42 mg/mL).

Conclusion

Together with ¹HNMR (1), FT-IR can be used to determine the presence of GHB free acid, GHB carboxylate, and GBL in aqueous solutions and aqueous-based forensic evidence. The methods developed involve minimal sample manipulation, allowing for the determination of the presence of GHB free acid in forensic samples while limiting the potential for GHB free acid to be generated in the course of analysis. Presumptive identification was based on IR measurement of the neat liquid sample in conjunction with second derivative spectral data processing to resolve the main carboxylic acid band of the free acid from GBL and water-based interferences. Confirmation was achieved using a subsequent dry down method requiring no solvent extraction, followed by FT-IR analysis of the residue.

As an analytical standard for GHB free acid is not commercially available, GHB free acid was prepared and isolated for use as a reference material for forensic testing. The method for preparation was based on a stoichiometric conversion of GHB’s sodium salt to the free acid in aqueous solution. Reaction yields were high (89–92%), with no detectable amounts of residual carboxylate or GBL observed. The procedure was shown to be rapid and reproducible, allowing for preparation at the time of use. The identity of the free acid reference material was confirmed by both FT-IR and ¹HNMR.
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