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A Novel Approach by SPME-GC/MS for the Determination of *gamma*-hydroxybutyric acid (GHB) in Urine Samples after Conversion into *gamma*-butyrolactone (GBL)

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Abstract

The quantitative determination of *gamma*-hydroxybutyric acid (GHB) in urine samples is very important to assess illicit intake or administration. To this end we evaluated several analytical methods: headspace gas-chromatography coupled to flame ionization detection (HS-GC/FID), headspace gas-chromatography coupled to mass spectrometry (HS-GC/MS), headspace gas-chromatography coupled to solid phase microextraction and mass spectrometry (HS-SPME-GC/MS). All these methods were endowed with a not sufficient sensitivity, and then we moved to solid phase microextraction coupled to gas-chromatography with mass spectrometry detection (SPME-GC/MS). At first, GHB was extracted from urine with an organic solvent and analyzed after derivatization. Under these conditions, however, there was a partial overlapping between the chromatographic peak of GHB and that of urea, also extracted by the organic solvent. Then we decided to change analytical approach and to convert GHB to *gamma*-butyrolactone (GBL), which is not an endogenous compound. A SPME method was optimized and validated for the determination of GBL. The limit of detection (LOD) of the method resulted to be 0.25 µg/mL for GBL, corresponding to 0.5 µg/mL for GHB. The lower limit of quantification (LLOQ) was 0.4 µg/mL for GBL and 0.8 µg/mL for GHB. The LLOQ of the method resulted 10 times lower than the endogenous level, thus allowing to distinguish between physiological conditions and exogenous assumption.

Keywords: *gamma-Hydroxybutyric acid (GHB); gamma-butyrolactone (GBL); HS-GC/FID; HS-GC/MS; HS-SPME-GC/MS; SPME-GC/MS*

Introduction

Background of GHB

gamma-Hydroxybutyric acid (GHB) is an endogenous short chain carboxylic acid, structurally related to *gamma*-aminobutyric acid (GABA), of which it is immediate precursor. It plays a role as a neurotransmitter and it is a central nervous system depressant [1-7]. GHB was first used as an anesthetic agent [6], but this indication was abandoned because of several adverse effects [8]; it was then introduced for the treatment of alcohol and opiate addiction [9-11]. During the 1970s and 1980s, GHB became popular at “rave” parties, causing many cases of abuse [12-15] and it was used by body builders for its supposed neuroendocrine effect, causing stimulation of growth hormone release [16]. A 0.5 g dose causes relaxation and disinhibition, a 1 g dose euphoric effects and a 2-3 g dose deep sleep [17-19]. At higher doses, GHB may lead to death [13]. Due to its effects, GHB is used to commit drug-facilitated sexual assaults as a “date rape” drug in combination with alcoholic beverages [19,20]. GHB is a controlled substance all over the world [21]. *gamma*-Butyrolactone (GBL), which is the intramolecular esterification product of GHB, is a solvent used in industrial applications such as paint removal and engine cleaning [8]. It is a precursor of GHB, not present as an endogenous compound, but if ingested it is rapidly hydrolyzed to GHB [22,23]; so GBL is also a controlled substance [21].

Analytical Methods for the Detection of GHB

Different analytical approaches have been reported in the literature for the determination of GHB in illicit preparations and biological samples, based on GC/MS, HPLC or FT-IR analysis [24-29]. Some of these methods are able to distinguish between GHB and GBL, while, in other cases, GHB is preventively converted into GBL in acidic conditions [30-32].

Purpose of this Work

In this frame, we were interested in comparing different literature methods by evaluating different techniques and derivatization and/or interconversion protocols, in order to establish a procedure easily applicable to the determination of GHB and GBL either in biological samples or in products seized on the illicit market. To this end we compared different techniques: headspace gas-chromatography coupled to flame ionization detection (HS-GC/FID), headspace gas-chromatography coupled to mass spectrometry (HS-GC/MS), headspace gas-chromatography coupled to solid phase microextraction and mass spectrometry (HS-SPME-GC/MS). All these methods resulted in a sensitivity which was not suitable to our purpose, and then we decided to move to solid phase microextraction coupled to gas-chromatography with mass spectrometry detection (SPME-GC/MS). The optimized method was then validated in urine samples coming from healthy volunteers and in *post mortem* samples.

Materials and Methods

Reagents Chemicals and Standards

All reagents were of analytical grade and were stored as indicated by the supplier. GHB, Ammonia, sulfuric acid, sodium chloride, pH 6 buffer, dichloromethane, *gamma*-crotonolactone, *delta*-valerolactone, N,O-Bis(trimethylsilyl) trifluoroacetamide 99% (BSTFA) and GHB-d_6 were purchased by Sigma-Aldrich, (Steinheim, Germany). GBL was obtained by LGC Standards (S. San Giovanni, Milano, Italy). Water (18.2 Ω cm^{-1}) was prepared by a Milli-Q System (Millipore, Darmstadt, Germany).

SPME fibers red fiber: polydimethylsiloxane (PDMS) coating with a thickness of 100 µm; black fiber: carboxen, polydimethylsiloxane (CAR/PDMS) coating with a thickness of 75 µm; white fiber: polyacrilate (PA) polar coating with a thickness of 85 µm; grey fiber: divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) with a thickness of 75 µm were purchased by Supelco (Sigma-Aldrich, Steinheim, Germany).

Standard stock solutions of GBL and GHB sodium salt and GHB-d_6 at concentrations of 1 mg/mL and 100 µg/mL were prepared in methanol.

Sample Preparation: 1 mL of blank urine was added with 150 µL of 97% H_2SO_4 when requested (acidified samples) and extracted with 5 mL of dichlorometane on a rotary shaker for 20 min. The mixture was then centrifuged (5 min; 5000 rpm), the organic phase was withdrawn and the solvent evaporated until a volume of 100 µL was reached. The sample was then moved to a 10 mL headspace vial and sodium chloride was added until a saturated solution was reached.

For the spiked samples 50 or 100 µL of a methanol solution of GHB (1 mg/mL) were introduced in an extraction tube. The solvent was evaporated and then the sample was prepared and extracted as described for the blank urine samples. The following samples were analyzed: sample A: blank solvent (water); sample B: blank urine from healthy volunteers containing endogenous GHB (Blank urine); sample C: blank urine samples acidified (Blank urine + H_2SO_4); sample D: blank urine samples acidified and spiked with GHB (Blank urine+GHB+H_2SO_4) at known concentration (50 µg $_{tot}$); sample E: urine sample coming from a case of GHB related death provided by the Institute of Legal Medicine of Palermo (Case); sample F: sample E acidified (Case+H_2SO_4); sample G: sample F spiked with GHB (Case+GHB+H_2SO_4) at known concentration (50 µg $_{tot}$); sample H: standard solution of GHB (100 µg $_{tot}$) in water (1 mL); sample J: standard solution of GBL (100 µg $_{tot}$) in water (1 mL); sample K: standard solution of GBL (10 µg $_{tot}$) in water (1 mL).

HS-GC/FID

HS-GC/FID analyses were performed on a Trace 2000 Thermo Electron GC system (Thermo Fisher Scientific, Waltham MA, USA) with a FID detector. The GC was equipped with a VF-624MS (30 m x 0.32 mm i.d., film thickness 1.8 µm) capillary column (Agilent Technologies) and an HSS 86.50 autosampler (DANI Instruments, Milano, Italy). Static HS conditions: incubation temperature, 100 °C (slow shaking); loop temperature, 120 °C; transfer line temperature, 150 °C; incubation time, 15 min.

The GC-FID system was operated under following conditions: injector temperature 160 °C; split mode; split ratio: 10/1; split flow: 33 mL/min; helium was used as the carrier gas at a flow rate of 2 mL/min; oven temperature program: 50 °C (3 min) - 150 °C, 10 °C/min; final isotherm; detector temperature: 250 °C; hydrogen and air were used as the detector gases at a flow rate of 35 mL/min and 350 mL/min respectively. Nitrogen at a flow rate of 30 mL/min was used as make-up gas.

HS-GC/MS

Analyses were carried out on a HP5890 Series II GC system (Agilent, Santa Clara, CA), with a split–splitless injection system operated in a split mode and an Agilent MSD HP5971 Detector operated in electron impact mode (70 eV). The GC was equipped with a capillary column VF-624MS (60 m, 0.25 mm i.d., film thickness 1.4 µm) (Agilent, Santa Clara, CA) and an HSS 86.50 autosampler (DANI Instruments, Milano, Italy).

Static HS conditions: incubation temperature, 100 °C (slow shaking); loop temperature, 120 °C; transfer line temperature, 150 °C; incubation time, 15 min.

The GC/MS system was operated under following conditions: injector temperature 200 °C; split mode; split ratio: 30/1; split flow: 33 mL/min; helium was used as the carrier gas at a flow rate of 1 mL/min; ; interface transfer line, 300 °C; ion source, 180 °C; oven temperature program: 70 °C - 150 °C, 5 °C/min.

Qualitative analysis was obtained in SCAN mode, mass range: 20 to 300 m/z. Quantitative determinations were carried out in SIM mode.

HS-SPME-GC/MS

The sample vial was equilibrated at 80 °C for 5 min. For adsorption, the needle of the SPME device containing the extraction fiber (SPME fiber assembly polyacrylate df 85 µm, Supelco, Sigma Aldrich, Steinheim, Germany) was inserted through the septum of the vial and the fiber was exposed to the headspace in the vial for 5 min. Finally, the SPME fiber with the absorbed compounds was introduced into the injection port of the GC/MS for 5 min to accomplish complete desorption of the analytes.

Analyses were carried out on a HP5890 Series II GC system (Agilent, Santa Clara, CA), with a split–splitless injection system operated in a split mode and an Agilent MSD HP5971 Detector operated in electron impact mode (70 eV). The GC was equipped with a capillary column VF-624MS (60 m, 0.25 mm i.d., film thickness 1.4 µm) (Agilent, Santa Clara, CA) and a manual holder for the SPME fiber. SPME fiber assembly Carboxen/Polydimethylsiloxane (CAR/PDMS) was used (Supelco, Sigma Aldrich, Steinheim, Germany).

The GC/MS system was operated under following conditions: injector temperature 280 °C; splitless mode with closing of the split valve 0.25 min form the insertion of the fiber; then split mode (split ratio: 30/1; split flow: 33 mL/min); helium was used as the carrier gas at a flow rate of 1 mL/min; ; interface transfer line, 300 °C; ion source, 180 °C; oven temperature program: 100 °C - 280 °C, 10 °C/min.

For qualitative analysis the MS detector was operated in SCAN mode, mass range: 20 to 300 m/z. Quantitative determinations were carried out in SIM mode.

SPME-GC/MS

For adsorption, the needle of the SPME device containing the extraction fiber (SPME fiber assembly polyacrylate df 85 µm, Supelco, Sigma Aldrich, Steinheim, Germany) was immersed in the sample.

Analyses were carried out on a HP5890 Series II GC system (Agilent, Santa Clara, CA), with a split–splitless injection system operated in a split mode and an Agilent MSD HP5971 Detector operated in electron impact mode (70 eV). The GC was equipped with a capillary column VF-624MS (60 m, 0.25 mm i.d., film thickness 1.4 µm) (Agilent, Santa Clara, CA) and a manual holder for the SPME fiber.

The GC/MS system was operated under following conditions: injector temperature 280 °C; splitless mode with closing of the split valve 0.25 min form the insertion of the fiber; then split mode (split ratio: 30/1; split flow: 33 mL/min); helium was used as the carrier gas at a flow rate of 1 mL/min; ; interface transfer line, 300 °C; ion source, 180 °C; oven temperature program: 100 °C - 280 °C, 10 °C/min.

Qualitative analysis was obtained in SCAN mode, mass range: 20 to 300 m/z. Quantitative determinations were carried out in SIM mode.

Results and Discussion

Optimization of the Analytical Method

First of all, the literature was carefully evaluated in order to choose the best conditions for GHB analysis. The most widespread method is based on extraction with an organic solvent and analysis of GHB by GC/MS after derivatization with BSTFA with a 5% phenyl-95% dimethylpolysiloxane capillary column. [27-32]. Extraction was initially accomplished by using ethyl acetate. This solvent, however, extracted also urea, which is not completely separated from GHB under the chromatographic conditions adopted. Therefore we decided to choose a more polar capillary column: VF-624MS (Agilent) with a stationary phase made of 6% cyanopropylphenyl/94% polydimethylsiloxane (PDMS). But the features of this column prevented us to directly inject the derivatized GHB extracted from the biological sample.

The analytical approach was then completely changed avoiding derivatization and transforming GHB into lactone. GBL is more volatile (b.p. 180 °C) respect to GHB (b.p. 295 °C) and it can be analyzed by headspace sampling reducing matrix effects. Different methods were compared in order to find the best conditions for GBL determination: HS-GC/FID; HS-GC/MS; HS-SPME GC/MS and SPME GC/MS. The most simple analytical approach was GC/FID coupled to static headspace sampling. The superimposition of the chromatograms of samples B,C,D, H is shown in Figure 1. No GBL is found in blank urine from healthy volunteers (B, green line), because the equilibrium is shifted toward the more stable form in vivo (GHB). GHB has a high boiling point and it is not evidenced by headspace sampling. In acidic conditions (C, light blue line), the equilibrium is shifted towards the lactone form and GBL is evidenced. The spiked sample (D, pink line) shows a more intense peak confirming that the peak effectively corresponds to GBL.

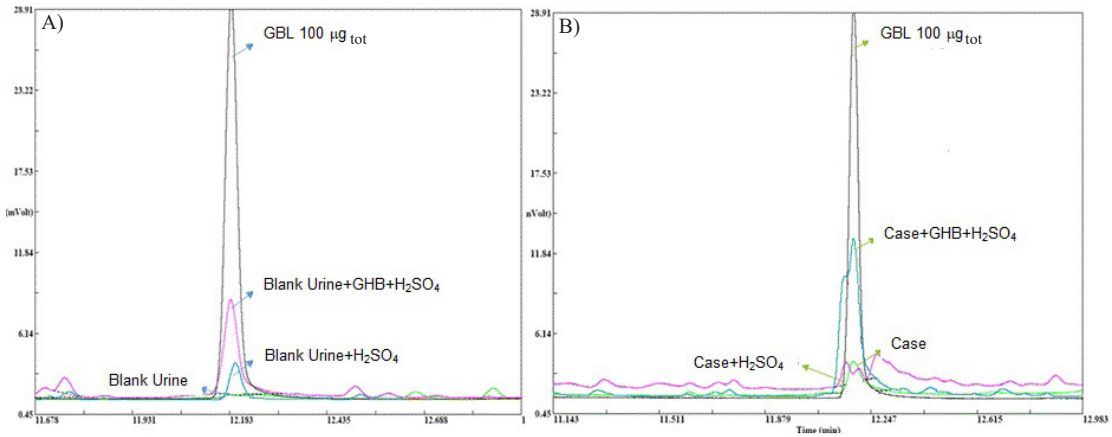

Figure 1: A) Superimposition of the HS-GC/FID chromatograms of samples B, C, D, J
B) Superimposition of the HS-GC/FID chromatograms of samples E, F, G, J

These preliminary analyses evidenced some critical issues: sensitivity was not enough to estimate endogenous levels of GHB. Moreover, in the case of autopsy samples, the situation was more complicated (Figure 1).

The autopsy sample (E, green line) shows a peak similiar to that of GBL, but this analyte has to be excluded because the equilibrium is shifted towards GHB in urine. The acidification of sample E (F, pink line) causes a deformation of the peak, probably due to interfering species. To identify these species we carried out a HS-GC/MS analysis.

The whole chromatogram (Figure 2) shows other peaks (butyric acid, valeric acid and isovaleric acid) with a retention time similar to that of GBL. The column coupled to the GC/MS instrument had a higher number of theoretical plates respect to the column of the GC/FID instruments; therefore a coelution of different compounds can be supposed in the GC/FID chromatographic conditions.

The increase of the peak of GBL from sample E to sample F is due to the shift of the equilibrium towards the lactone form after acidification. The increase of the peaks of valeric, isovaleric and butyric acid after acidification is due to the protonation of the carboxylic acids and their major distribution in the organic phase. The HS-GC/MS method showed a higher sensitivity respect to the HS-GC/FID one, but nevertheless insufficient for the determination of endogenous levels of GHB. Therefore, we decided to move to solid phase microextraction (SPME) technique. At first, we applied HS-SPME-GC/MS technique. Four different fibers were evaluated. Red fiber: polydimethylsiloxane (PDMS) coating with a thickness of 100 µm; black fiber: carboxen, polydimethylsiloxane (CAR/PDMS) coating with a thickness of 75 µm; white fiber: polyacrilate (PA) polar coating with a thickness of 85 µm; grey fiber: divinylbenzene/carboxen/

polydimethylsiloxane (DVB/CAR/PDMS) with a thickness of 75 µm. K_{fs} values (partition coefficient for analyte between coating and sample matrix) for GBL with the different fibers were evaluated by incubating 100 µL of a methanol solution (1 mg/mL) of GBL (100 μg_{tot}) diluted in 1 mL of water at 70 °C for 15 min. Desorption was then carried out in the injector for 5 min. The results are reported in Figure 3A. As it is evident, the black fiber provided the highest adsorption.

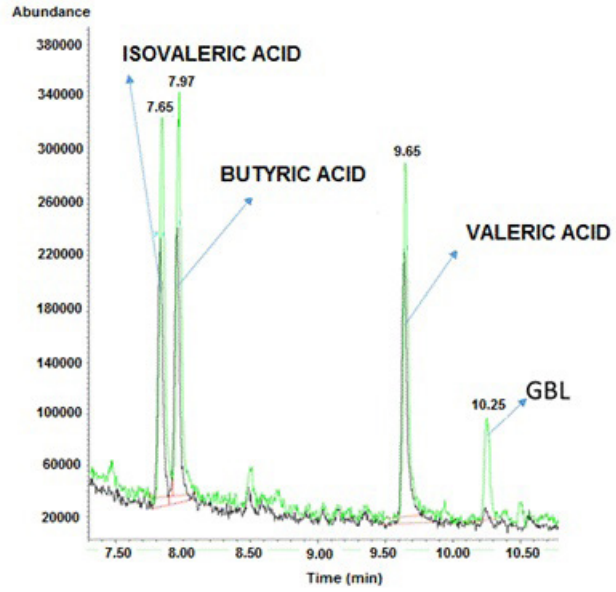

Figure 2: Superimposition of HS-GC/MS chromatograms of sample E (black line) and sample F (green line)

The fibers were directly tested immerging them into the samples and the results reproduce those obtained for the adsorption.

The fibers were also tested for the adsorption of GHB by immersion, but the results were not satisfactory, so the conversion of GHB into GBL proved to be mandatory to achieve a suitable extraction recovery. To compare extraction recovery obtained by HS-SPME or SPME by immersion, two samples of urine spiked with 10 μg_{tot} of GBL were prepared and incubated at 35 °C for 15 min. In Figure 3B the comparison is shown. As it is evident, the immersion of the fiber in the sample provided a higher adsorption respect to headspace sampling.

The optimization of the chromatographic method focused on the desorption of the analyte from the fiber. Several parameters were taken into account: liner geometry, injection (split/splitless), control of the splitting valve, desorption temperature. The shape of the peak, which is an index of the efficiency of the chromatographic system, was evaluated to optimize these parameters. Different liners were taken into account: 4 mm internal diameter liner, 2 mm internal diameter liner, single tapered liner with an internal diameter of 4 mm and double tapered liner with an internal diameter of 4 mm. The best chromatographic efficiency was obtained by using the liner with an internal diameter of 2 mm without restrictions. Several desorption experiments were carried out either in split (split ratio 30:1, spilt flow 30 mL/min) or in splitless mode. A little gain in terms of efficiency was obtained with the split mode, but sensitivity was dramatically reduced. So we decided to operate in splitless mode and we evaluated the influence of the closing time of the splitting valve on the chromatographic efficiency. Three times were tested (0.25, 1 and 2 min), maintaining the time of desorption constant (5 min); the best results were obtained with a closing time of 0.25 min.

Moreover four different injection temperatures were evaluated for the desorption of the analytes: 200, 250, 280, 300 °C. The best one was 250 °C; in fact, this temperature allows the optimal desorption without damaging the SPME fiber.

Once we established the black fiber as the best one for the extraction of GBL from urine samples, the microextraction temperature of the sample, the exposition time of the fiber and the desorption time of the analyte from the fiber were considered. Experiments were carried out on GBL with a concentration of 10 µg/mL. Five different incubation temperatures were tested: 5, 25, 30, 35, 40 °C. The fiber was immersed for 15 min and the desorption time was 5 min. We observed that the adsorption of GBL increased with temperature (Figure 3C).

Incubation temperature was set at 35 °C because, at this temperature, the analyte was efficiently extracted, and rapidly adsorbed without any degradation of fiber.

Five adsorption times were taken into account: 5, 15, 20, 25, 35 min. The incubation temperature was fixed at 35 °C and the desorption time was 5 min. A direct proportionality was found between the time of exposure of the fiber and GBL extraction (Figure 3D).

It is evident that the adsorption is not complete even after 35 min. On the other hand, a time of adsorption of 15 min is enough to achieve a suitable extraction recovery without excessively increasing the time of analysis.

The desorption time was then evaluated; five different desorption times were considered: 0.5, 2.5, 5, 7.5, 10 min. The influence of the desorption time on the GBL peak height is reported in Figure 3E. A maximum of peak height is reached with a desorption time of 5 min.

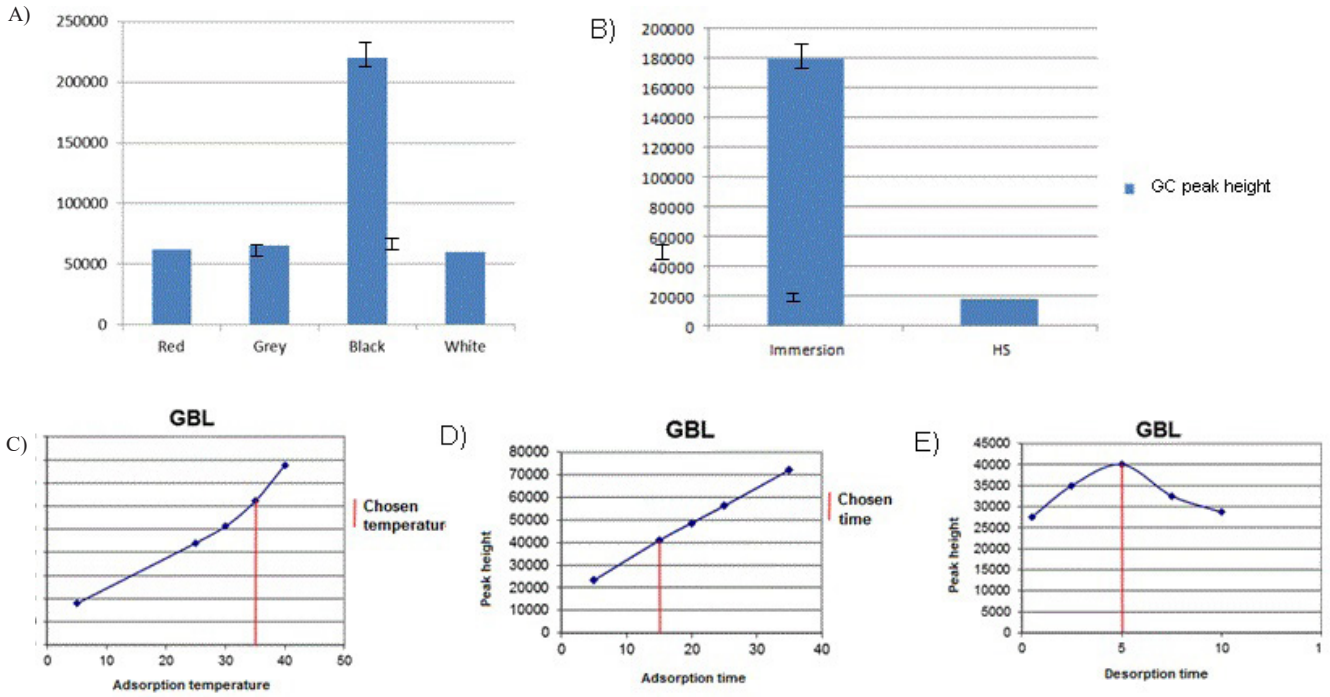

Figure 3: A) Adsorption of GBL on different SPME fibers. Red fiber: polydimethylsiloxane (PDMS) coating with a thickness of 100 µm; black fiber: carboxen, polydimethylsiloxane (CAR/PDMS) coating with a thickness of 75 µm; white fiber: polyacrilate (PA) polar coating with a thickness of 85 µm; grey fiber: divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) with a thickness of 75 µm; B) Comparison between immersion and HS sampling on a black fiber; C) Adsorption of GBL as a function of the incubation temperature; D) Extraction of GBL as a function of adsorption time; E) Extraction of GBL as a function of desorption time

The optimized parameters for SPME analysis of GBL are summarized in Table 1.

Injection temperature	280 °C
Liner geometry	2mm without restrictions
Injection system	splitless
Closing time of the splitting valve	0.25 min
Incubation temperature	35 °C
Adsorption time	15 min
Desorption time	5 min

Table 1: Optimized parameters for the SPME analysis of GBL

To carry out a quantitative determination, the choice of a suitable internal statndard (IS) was mandatory. delta-Valerolactone showed a good affinity for the SPME fiber and the chromatographic peak was well separated from that of GBL (Figure 3).

Another important parameter to be considered is sample preparation. The conversion of GHB into GBL requires the acidification with concentrated sulfuric acid. In order to avoid the damage of the fiber, the sample has to be neutralized before the immersion of the fiber. Summarizing, sample preparation of the standard solutions of GHB is as follows: varying amounts of a methanol solution of GHB (1 mg/mL) are introduced into a vial and the solvent is evaporated. A 1 mg/mL methanol solution of delta-valerolactone (18 µL) and blank urine (1 mL) are added: the mixture is saturated with sodium chloride and then 150 µL of 97% sulfuric acid are added. The mixture is shaken on a rotary shaker for 30

min, 500 µL of 30% ammonia (pH 7-7.5) and 1 mL of pH 6 buffer are added, then the fiber is immersed in the mixture.

Validation of the Analytical Method

The optimized SPME-GC/MS method for the determination of GBL in urine sample was validated to meet the forensic analysis requirements.

Linearity was studied by analyzing five non-sequential concentrations of GBL (1.4, 2.8, 13.8, 20.6, 27.5, 41.3, 55.0 μg_{tot}) in triplicate. The linearity of the method was adequate in all the range. The equation and correlation coefficient were $y=0.1691x-0.0388$ and $R^2=0.9945$ respectively.

LLOQ was considered as the lowest concentration at which linearity was still satisfied and the signal to noise ratio was at least ten. LLOQ resulted to be 0,4 µg/mL for GBL corresponding to 0.80 µg/mL of GHB.

LOD was assessed by progressively diluting the solution prepared for the determination of the LOQ until a signal to noise ratio of three was reached. It resulted to be 0.25 µg/mL for GBL corresponding to 0.50 µg/mL of GHB.

Accuracy was evaluated as % recovery according to the following formula: %REC=(analytical concentration/real concentration)×100. The analytical concentration was calculated from the RA values (RA=Area of the analyte/Area of the IS) on the basis of the linearity equations. The mean % REC was 101. 5 %.

Repeatability was evaluated analyzing six samples at different concentrations of GBL (0.5, 5, 7.5, 10, 15, 20 µg/mL) in the same day: the obtained % CV was 6.6%.

Yield of Conversion GHB/GBL

As the selected method requires the conversion of GHB into GBL, the yield of the transformation was calculated by preparing three GBL samples (6 µgtot) and comparing them with three acidified samples of GHB-d6 (7.5 µgtot, Figure 2). The quantity of GHB-d6 was chosen because is equivalent to 6 µgtot of GBL. The results are reported in Table 2. The mean yield was 62.8 %.

	Sample	RA	μg_{tot} Analyte	μg_{tot} GBL after acidification	Yield %
GBL	1	0,1722	6.0		
	2	0,1672	6.0		
	3	0,1710	6.0		
GHB-d_6	4	0,2779	7.5	3.7	62.0
	5	0,2634	7.5	3.8	63.5
	6	0,2714	7.5	3.8	63.0

Table 2: Conversion of GHB into GBL. $RA=A_{analyte}/A_{IS}$

Conclusions

Several different experimental conditions were examined for the qualitative and quantitative determination of GHB in urine samples. A SPME method by immersion of the fiber was optimized and validated for the determination of GBL: the LLOQ of the method resulted 10 times lower than the endogenous level, thus allowing physiological conditions to be distinguished from exogenous assumption. The method was applied to a case of GBL related death.

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