

Determination of gamma-hydroxybutyric acid in dried blood spots using a simple GC-MS method with direct “on spot” derivatization

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Abstract The objective of this study was the development of an accurate and sensitive method for the determination of gamma-hydroxybutyric acid (GHB) in dried whole blood samples using a GC-MS method. The complete procedure was optimized, with special attention on the sample pretreatment, and validated. Therefore, dried blood spots of only 50 μl were prepared and, after the addition of internal standard GHB-d6, directly derivatized using 100 μl of a freshly prepared mixture of trifluoroacetic acid anhydride and heptafluorobutanol (2:1). The derivatized extract was injected into a gas chromatograph coupled to a mass spectrometer (GC-MS), operating in the electron impact mode, with a total run time of 12.3 min. Method validation included the evaluation of linearity, precision, accuracy, sensitivity, selectivity, and stability. A weighting factor of $1/x^2$ was chosen and acceptable intra-batch precision, inter-batch precision, and accuracy were seen. The linear calibration curve ranged from 2 to 100 $\mu\text{g/ml}$, with a limit of detection of 1 $\mu\text{g/ml}$. Our procedure, utilizing the novel approach of direct “on spot” derivatization followed by analysis with GC-MS, proved to be reliable, fast, and applicable in routine toxicology.

Keywords Gamma-hydroxybutyric acid (GHB) · Gas chromatography–mass spectrometry (GC-MS) · Dried blood spots (DBS) · Direct derivatization · Method validation

Introduction

Gamma-hydroxy butyric acid (GHB) is a short-chain fatty acid synthesized in the early 1960s as an anesthetic structure analogue of gamma-amino butyric acid (GABA). Later on, it was found to be endogenously present in mammalian brain tissue [1–3]. The function of GHB in humans is not completely understood, but experimental evidence suggests that it may act as a neuromodulator or neurotransmitter [4]. GHB and its precursors gamma-butyrolacton (GBL) and 1,4-butanediol (1,4-BD), which are converted into GHB within minutes after oral ingestion, are sold as a dietary supplement and used by bodybuilders as it is believed that GHB has a stimulatory effect on growth hormone production [5, 6]. Although it has been abandoned as anesthetic agent in most countries because of side effects such as seizures and coma, it is still registered in Germany (Somsanit®). The sodium salt of GHB, known under the international drug name sodium oxybate, has a role in the treatment of narcolepsy with cataplexy (Xyrem®) and of opiate and alcohol withdrawal (Alcover®) [7–11].

Currently, GHB as well as its precursors are popular as a party or club drug (liquid ecstasy) and appear occasionally in drug-facilitated sexual assaults (DFSA). This is due to their effects and chemical properties as they are colorless, odorless liquids which can be easily mixed with other liquids [11–14]. GHB is mostly misused in combination with other drugs, such as alcohol, ecstasy (MDMA, XTC), cocaine, amphetamines, and cannabis [12, 13, 15]. The most commonly reported effects of GHB abuse are euphoria, increased sexuality, well-being, and tranquility, while an overdose results in seizures, respiratory depression, coma, and sometimes even death [15–17]. The range

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between the desired effects and an overdose is narrow, frequently resulting in (non-)fatal intoxications in humans, as described in several case reports [18–24].

Consequently, the identification and correct quantification of GHB is important in forensic and clinical toxicology. Many previously reported analytical methods detect GHB in different biological matrices and involve the use of gas chromatography (GC) [25–27], but also liquid chromatography (LC) [28, 29] and capillary zone electrophoresis [30]. Also, the determination of GHB through headspace solid phase microextraction and dynamic extraction has recently been published [31, 32].

However, the determination of GHB in biological matrices remains an analytical challenge for several reasons. First, GHB is a small polar molecule, making its extraction and the direct detection with GC difficult. Hence, two approaches are generally used: Besides the conversion of GHB in acidic conditions to GBL, which is more easily extracted from the biological matrix, the carboxyl and hydroxyl groups can be derivatized, resulting in a more volatile and less polar compound. As derivatization technique, silylation is mostly used, but also alkylation and acylation have previously been described [25, 27]. Furthermore, GHB occurs naturally in blood and urine, so no blank matrix is available and positive samples must be carefully interpreted. To enable the differentiation between exogenously administered and endogenous GHB, cutoff levels have been proposed by several authors. These are currently set at 4 or 5 $\mu\text{g/ml}$ for blood samples and 6 or 10 $\mu\text{g/ml}$ for urine samples [33–36]. In addition, once orally ingested, GHB is rapidly metabolized, with a reported plasma half-life of <1 h. As this limits the detection window, plasma samples must be taken within 6 h and urine samples within 12 h (or better both within 6 h) after ingestion [18].

In this study, we use dried blood spots (DBS) to improve the detection and quantification of GHB by facilitating sample collection. A DBS is capillary whole blood obtained by a finger or heel prick and collected on a filter paper. This sampling technique ensures an easy and rapid collection of a representative sample without specific handling and storage requirements. These advantages over a venipuncture make it a cost-effective choice for the collection, transport, and storage of blood samples [37, 38]. The DBS sampling method, originally used in newborn screening for metabolic disorders, is currently applied for the determination of various compounds such as biomarkers and is promoted for therapeutic drug monitoring. Already several compounds can be detected in DBS, the most common being anti-malaria drugs, immunosuppressive drugs, anti-epileptics, antibiotics, and anti-diabetics [38]. Inherent to DBS sampling is the small sample volume available, ranging from 10 to 200 μl , compared to 1 ml or more

obtained by a venipuncture. Although this may be an advantage when the collection of larger amounts of whole blood is limited, such as in neonates and children, these small amounts may impose an analytical challenge, requiring an efficient sample pretreatment and a sensitive detection [39, 40].

The determination of GHB in DBS may be interesting in situations where there is a suspicion of illicit use of GHB or one of its precursors, for example in the case of driving under the influence of drugs (DUID) or a presumed DFSA. As mentioned above, the short half-life of GHB implies a limited detection window and, consequently, a rapid collection to obtain a representative sample. A delay caused by the need for a venipuncture by medical staff may bring the blood levels of GHB under the established cutoff levels [41]. Moreover, no extraction step is necessary as DBS can be directly derivatized, minimizing the sample preparation and reducing the sample turnaround time. Besides the advantages of a rapid collection, also storage of whole blood samples as DBS may be of interest as this may avoid *in vitro* formation of GHB, which has previously been reported [35, 42]. Finally, even though DBS analyses are most commonly performed using LC-MS/MS, we opted to use GC-MS for several reasons. First of all, GC is a common and available technique in forensic laboratories, proven to be suitable for the detection of derivatized GHB with good sensitivity [25]. Secondly, given the low molecular weight of GHB, adequate and sensitive detection following liquid chromatography requires extensive sample pretreatment, involving an extraction step and/or derivatization to enhance selectivity. Therefore, the aim of this study was to develop and validate a GC-MS method for the identification and quantification of GHB in DBS, based on a new procedure, involving direct derivatization of GHB “on spot”, ensuring a minimal, economic, and less time-consuming sample pretreatment.

Materials and methods

Reagents

Chemicals

GHB (sodium salt) as powder and as a 1 mg/ml solution in methanol, as well as a 1 mg/ml solution in methanol of the internal standard (IS) GHB-d6 (sodium salt), were obtained from LGC standards (Molsheim, France). The derivatization reagents heptafluorobutanol (HFB-OH) and trifluoroacetic acid anhydride (TFAA) were purchased from Sigma Aldrich (Steinheim, Germany). Methanol and ethyl acetate, both of suprasolve quality suitable for GC analysis, were delivered by Merck (Darmstadt, Germany).

Stock and working solutions

Stock and working solutions of GHB were prepared by dissolving 10 mg of the base in 1 ml of methanol, followed by appropriate dilution to obtain 1 and 0.1 mg base/ml methanolic solutions. Quality controls for all analyses were obtained from the commercially available stock solution of 1 mg Na-GHB/ml methanol. For the IS, a solution of 0.05 mg base/ml was prepared starting from a 1 mg Na-GHB-d6/ml stock solution in methanol. All solutions were stored at $-20\text{ }^{\circ}\text{C}$.

Materials

Protein saver cards number 903, used as sampling paper, were kindly provided by Whatman (ref. no. WHA10334885, Dassel, Germany). The automatic lancets for capillary blood collection were purchased from Becton Dickinson (ref. no. VAC366594, Franklin Lakes, USA), while the 50- μl precision capillaries were obtained from Servo-Prax (Wesel, Germany). The centrifuges used were a MSE Mistral 2000 (Beun de Ronde Serlabo, Anderlecht, Belgium) and a 5804R Eppendorf (Hamburg, Germany). Evaporation under nitrogen was conducted in a TurboVap LV evaporator from Zymark (Hopkinton, MA, USA).

DBS sample collection

Two methods are commonly used to obtain capillary whole blood on a filter paper. The drop of blood can be collected directly on the filter paper or with the aid of a precision capillary. In this study, we opted to use the latter method in which a drop with a fixed volume is spot onto the paper. As the complete drop can be excised instead of punching out a disk from it, the influence of sample volume, hematocrit, and sampling technique is minimized [38].

In the procedure to obtain a DBS, the hand is first cleaned and held down or warmed for a few minutes. With the help of an automatic lancet, the fingertip is pricked. While the first drop is wiped off with a sterile piece of cloth because of the presence of tissue fluid, the following drops are collected in a 50- μl precision capillary. Then, once completely filled, the entire capillary is placed in the center of two concentric circles pre-printed on a Whatman 903 filter paper. The inner circle (10-mm diameter) must be entirely filled with blood, but blood may not pass the outer circle line (15-mm diameter), which was used for excision of the DBS. Although the blood is spot on just one side, both sides of the filter paper must be colored. After visual inspection of the DBS, the analyses can start [38].

For method development and validation, we used 50- μl spots of venous whole blood from healthy non-user volunteers with endogenous GHB concentration below the

established lower limit of quantification (LLOQ) and preserved for a maximum of 1 week at $4\text{ }^{\circ}\text{C}$. The DBS are dried for a minimum of 4 h at ambient temperature and subsequently analyzed or preserved in a sealable plastic bag at room temperature or $-20\text{ }^{\circ}\text{C}$ until analysis.

Optimization of the DBS sample preparation

First, 10 μl of the IS solution was applied to a DBS and left to dry for 15 min. Subsequently, the spot was completely excised following the outer circle line, placed in a test tube, and a freshly prepared mixture of TFAA and HFB-OH was added, followed by sonication for 5 min. Sabucedo and Furton [27] described the use of 1 ml of this mixture to derivatize GHB in aqueous samples (drinks) at $85\text{ }^{\circ}\text{C}$ for 30 min, while we desired the derivatization of GHB in a biological matrix. For this purpose, the main critical parameters for derivatization, such as the amount of reagent, temperature, and time, were thoroughly evaluated. Therefore, different amounts of derivatization mixtures were tested, respectively 75, 100, and 125 μl of a TFAA and HFB-OH (2:1) solution. Also, derivatization temperatures varying from room temperature to 60, 70, 85, and $100\text{ }^{\circ}\text{C}$ and derivatization times ranging from 5 to 30 min were tested. For each condition, at least three DBS spiked with GHB at a 10- $\mu\text{g/ml}$ concentration level were analyzed, and the resulting absolute peak areas of derivatized GHB were compared.

After the derivatization step, the DBS was cooled down by centrifugation for 5 min at $4\text{ }^{\circ}\text{C}$ and evaporated to dryness under a gentle stream of nitrogen at $25\text{ }^{\circ}\text{C}$ to remove excess of derivatization reagent. The dried sample was redissolved in 200 μl of ethyl acetate, sonicated, and centrifuged for 5 min at $1,600\times g$. Eighty-five microliters was transferred, centrifuged again, and, finally, 50 μl was transferred to a vial, of which 1 μl was injected into the GC-MS.

The analytical procedure

Samples were analyzed on an Agilent 6890 GC system coupled to a 5973 mass-selective detector. Splitless injections were performed automatically at an injection temperature of $250\text{ }^{\circ}\text{C}$ and a purge time of 1.5 min, and helium was used as carrier gas at a constant flow rate of 1.3 ml/min. A $30\text{ m}\times 0.25\text{ mm i.d.}\times 0.25\text{ }\mu\text{m}$ Varian VF-5-MS column (Varian, Middelburg, The Netherlands) was used. The temperature program was started at $60\text{ }^{\circ}\text{C}$ for 1.5 min, ramped at $10\text{ }^{\circ}\text{C/min}$ to $110\text{ }^{\circ}\text{C}$, then raised by $50\text{ }^{\circ}\text{C/min}$ to $300\text{ }^{\circ}\text{C}$, which was held for two more minutes for optimal column performance and maintenance, resulting in a total run time of 12.3 min. The transfer line temperature and ion source temperature were set at 280 and $230\text{ }^{\circ}\text{C}$, respectively. MS quadrupole temperature

was set at 150 °C and ionization energy of 70 eV was used. The mass spectrometer operated in the selected ion monitoring (SIM) mode using electron impact ionization (EI) for quantification of GHB and GHB-d6. By running standards in full scan, typical mass spectra were obtained and following ions were monitored in the SIM mode: m/z 155, 183, 227, and 242 for derivatized GHB and m/z 161, 189, 231, and 245 for derivatized GHB-d6 (underscored ions represent the quantifier ions as these had the highest abundance; the other ions were selected as qualifier ions).

Validation

The following criteria were evaluated to validate the method: linearity, precision, accuracy, sensitivity, selectivity, and stability [43–45].

Linearity, precision, accuracy, and sensitivity

As no blank matrix is available, fresh venous human whole blood withdrawn on EDTA as anticoagulant was used to prepare the calibration standard solutions. The concentration of endogenous GHB was tested to be below the LLOQ of the method (<2 µg/ml), and the ratio of GHB to GHB-d6 was always lowered with the mean ratio of the zero samples (blank whole blood + IS, in duplicate) measured on each calibration day.

A five-point calibration curve was constructed six times on six different days. Each day, a blank, 2, 10, 25, 50 and 100 µg/ml solution of GHB in whole blood was prepared, as well as the QC solutions at low (2 µg/ml), medium (10 µg/ml) and high (100 µg/ml) concentration levels, covering the whole calibration range. From each solution, 50-µl spots were made and left to dry at room temperature for a minimum of 4 h, followed by analysis. The resulting data were statistically evaluated and weighted if necessary based on the sum% relative error (RE) and the % RE plot versus concentration, where % RE is the concentration found lowered with the nominal concentration, divided by the nominal concentration and multiplied by 100. Linearity was assessed by performing Fisher's test [43, 46].

Intra-batch precision was assessed by replicate analysis of spiked samples ($n=5$ at low, medium, and high concentration levels) in a single day; inter-batch precision was evaluated by determination of spiked samples per concentration on 6 days. Precision was measured by calculating the relative standard deviation (RSD, SD divided by the mean and multiplied by 100). Also, accuracy was determined for each concentration level, calculated by the percent deviation from the nominal concentration.

For the evaluation of sensitivity, the limit of detection (LOD) and the limits of quantification (LLOQ and ULOQ) were determined. The LOD was estimated as the minimum

concentration of GHB with a signal-to-noise ratio equal or larger than 3, so with reliable differentiation of the background noise and in compliance with the identification criteria [46, 47]. The lowest and highest concentrations of GHB still measured with acceptable precision (RSD <20% for LLOQ, 15% for ULOQ) and accuracy (80–120% for LLOQ, 85–115% for ULOQ) were chosen as the LLOQ and ULOQ, respectively.

Furthermore, possible dilution of the final extract of samples above the ULOQ was investigated. Therefore, human whole blood was spiked at 200 µg/ml (twice the ULOQ, in duplicate) and 50-µl spots were processed as described above ($n=3$). Tenmicroliters of the final derivatized extract was diluted to 100 µl with ethyl acetate, analyzed by GC-MS, and corrected for the dilution factor. Carryover was also tested by injecting the highest concentration level of the calibration curve, followed by three blank (ethyl acetate) injections.

Selectivity

The possible contribution of the isotopically labeled IS was assessed by analyzing both blank and zero (blank whole blood spiked with IS) samples. Furthermore, we investigated the possible interference of structural analogues such as beta-hydroxybutyric acid, alpha-hydroxybutyric acid, and GABA and the precursors GBL and 1,4-BD (spiked at 100 µg/ml), as well as the mainly co-ingested club and date rape drugs (alcohol, cocaine, benzoylcegonine, Δ^9 -tetra-hydrocannabinol (THC), 11-nor-9-carboxy-THC, ketamine, flunitrazepam, XTC, and amphetamine, spiked at or well above the concentrations typically found in abusers). At least six different sources of whole blood were spiked and analyzed in order to compare the ratio of GHB to GHB-d6 of blank samples, spiked at a known GHB concentration, with that of the samples containing the interferences.

Stability

As the filter paper matrix is expected to stabilize most analytes in DBS, we evaluated the stability of GHB in DBS stored at different conditions [37]. Therefore, three separate solutions in whole blood at both low (5 µg/ml) and high (100 µg/ml) concentration levels were prepared, and the resulting spots were preserved in a sealable plastic bag at room temperature for 1 week, at 4 °C for 24 h, and at -20 °C for 14 days. For the first 48 h at room temperature, DBS were analyzed after 2, 4, 8, 12, 24 and 48 h to assess the influence of drying time. The mean percentage found needs to be within 85–115% of the results obtained when analyzed after 4 h of drying as this is the minimum drying time recommended by the

manufacturer and therefore chosen as the reference time point.

To assess the stability of the processed samples, extracts (low and high concentration levels, in triplicate) were re-injected after 24 h at room temperature and after storage for a minimum of 1 week at $-20\text{ }^{\circ}\text{C}$ (per concentration level, in duplicate). The ratio of GHB to GHB-d6 was compared to that of the directly analyzed extract. Stability of stock solutions at two concentration levels ($n=3$) was assessed over 14 days at $-20\text{ }^{\circ}\text{C}$ and after three freeze–thaw cycles.

Application

The described procedure was applied to blood samples of two young men suspected of GBL intake, along with other drugs, to evaluate the routine applicability and the easiness of the method in a laboratory setting. The two young men were found on the street under influence—one was in critical condition—and were brought to a nearby hospital. Blood and urine samples were taken and sent to our laboratory for analysis of drugs and alcohol. As soon as the blood samples arrived at the laboratory, DBS were prepared and stored until analysis. Along with the DBS analysis, the blood samples were also subjected to routine toxicological analysis, involving screening by enzymatic assays and confirmation of positive results by high-performance liquid chromatography–diode array detection (HPLC-DAD) and GC-MS.

Results and discussion

Optimization of the DBS sample preparation

Inherent to DBS sampling is the small sample volume available, necessitating an efficient sample preparation [39]. Therefore, different procedures were tested and compared to obtain the optimal conditions of extraction and derivatization of GHB in order to work as sensitive as possible in an easy and time-saving way.

Optimization of the sample preparation resulted in the setup of a quick and efficient protocol. Direct derivatization took place by adding $100\text{ }\mu\text{l}$ of a freshly prepared mixture of TFAA and HFB-OH (2:1) to a test tube containing the excised DBS. Then, the test tube was sonicated for 5 min, ensuring the distribution of the derivatization reagent [48], and placed in a heating block at $60\text{ }^{\circ}\text{C}$ for 10 min. After cooling down, the sample was dried under a gentle stream of nitrogen at $25\text{ }^{\circ}\text{C}$. Next, the DBS was redissolved in $200\text{ }\mu\text{l}$ of ethyl acetate, sonicated, and centrifuged two subsequent times, followed by transfer of an aliquot of the supernatant to a vial, of which $1\text{ }\mu\text{l}$ was injected into the GC-MS. The overall procedure from receipt of a DBS to a quantitative result can be completed in $<2\text{ h}$.

As the polarity and the low molecular weight of GHB require derivatization or conversion to GBL prior to GC analysis, we chose direct derivatization instead of conversion, thus avoiding extraction and resulting in an easier, less

Fig. 1 Representative chromatograms of derivatized extracts of a blank DBS spiked with IS at $10\text{ }\mu\text{g/ml}$ (a) and a DBS spiked with GHB at $2\text{ }\mu\text{g/ml}$ (LLOQ) and IS at $10\text{ }\mu\text{g/ml}$ (b), analyzed with GC-MS in the EI-SIM mode

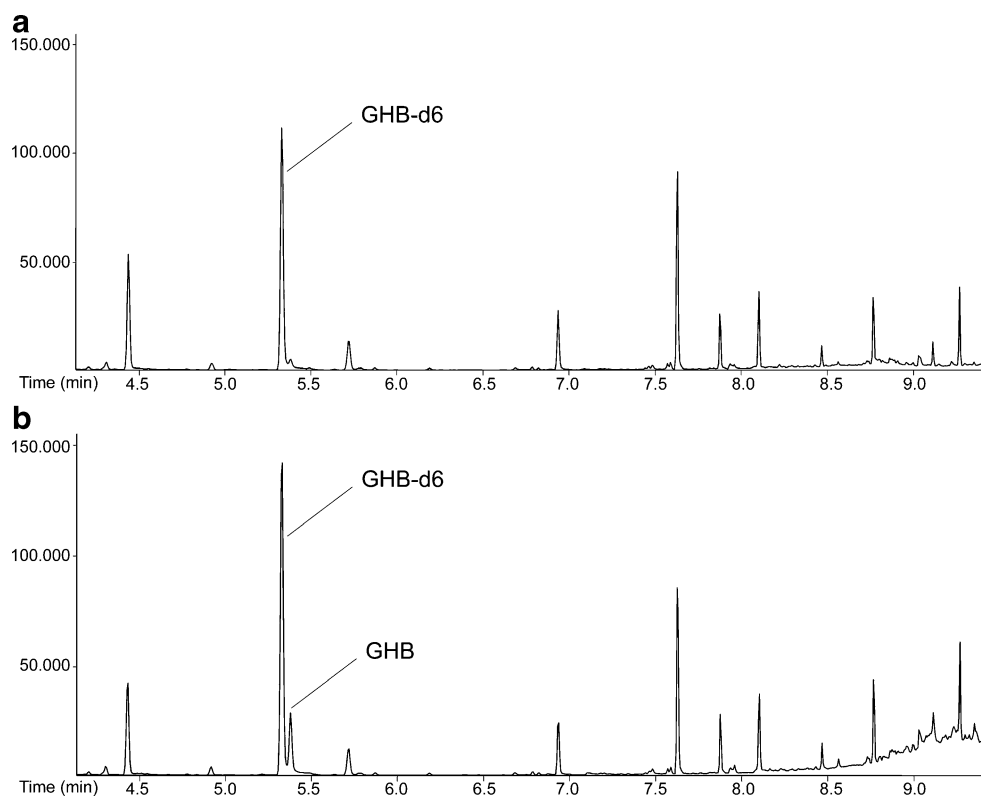


Table 1 Validation data for the determination of GHB in DBS using GC-EI-MS: calibration and sensitivity data

LOD ($\mu\text{g/ml}$)	LLOQ ($\mu\text{g/ml}$)	Slope ($n=6$), mean \pm SD (95% CI)	Intercept ($n=6$), mean \pm SD (95% CI)	Linear range ($\mu\text{g/ml}$)	R^2
1	2	0.087 \pm 0.004 (0.083–0.090)	-0.012 \pm 0.03 (-0.036–0.011)	2–100	1.000

time-consuming sample preparation. Apart from a method previously published by our group, only one other published method utilizes direct derivatization of GHB [26, 27]. However, both methods have as a major drawback the requirement of a considerably larger amount of derivatization reagent, around 1 ml for each sample, while here, only 100 μl is needed, contributing to a more economic and environment-friendly procedure. Déglon et al. recently published a method for the determination of antidepressants in DBS, with extraction using 500 μl 0.02% triethylamine in butyl chloride, in combination with direct derivatization using 100 μl derivatization reagent [49]. However, to the best of our knowledge, our procedure is the first where derivatization reagents are applied directly “on spot” without the use of any extraction solvent.

Analytical procedure and validation

The optimized conditions for sample pretreatment (heating the excised DBS for 10 min at 60 °C with 100 μl of a freshly prepared mixture of TFAA and HFB-OH (2:1), followed by drying and redissolving in 200 μl ethyl acetate; see “[Optimization of the DBS sample preparation](#)”) and analysis by GC-MS (as described in “[The analytical procedure](#)”) were carried out for the validation of the method.

Linearity, precision, accuracy, and sensitivity

To assess linearity, a blank, two zero (blank whole blood + IS), and five calibration samples were analyzed on six different days. Representative chromatograms of the injection of a derivatized extract of a blank DBS and a DBS containing GHB in a concentration at the lower end of the calibration curve (LLOQ) are shown in Fig. 1a, b, respectively. Table 1 summarizes the calibration and

sensitivity data. Ratios of GHB to GHB-d6 were calculated by dividing the peak area of GHB by the peak area of GHB-d6 and were lowered with the mean ratio of the zero samples. Results were statistically evaluated and a weighting factor $1/x^2$ was applied to the linear calibration curves. Also, the zero value was included in the 95% confidence interval of the y -intercept, indicating the absence of a constant error. As indicated in Table 2, overall intra- and inter-batch precision and accuracy were below 15% and below 20% at LLOQ level.

To evaluate the ability to dilute samples with a GHB concentration above the ULOQ, replicate DBS of a 200 $\mu\text{g/ml}$ solution were analyzed as described in “[Materials and methods](#).” Following a tenfold dilution of the derivatized extract, the concentration was back-calculated using daily calibration curves, and precision and accuracy were found to be within the acceptance limits of 15%. No carryover was seen following injection of a sample with GHB spiked at ULOQ level (<0.085%).

Selectivity

The fact that preliminary studies indicated a small interference at the retention time of GHB by injection of a derivatized extract of a blank filter paper, combined with the endogenous presence of GHB in whole blood, rendered it impossible to prove the lack of response of the blank matrix. However, as the paper signal and the signal of the used whole blood had a peak area below 10% and 20% of that of the LLOQ, respectively, no unacceptable interferences were seen [45]. No unacceptable interferences were seen at the retention time of the IS either. When analyzing both blank and zero samples (the latter corresponding to blank + IS), no significant difference was seen in the mean response of GHB.

To further evaluate selectivity, six different sources of whole blood were spiked with a combination of structure

Table 2 Validation data for the determination of GHB in DBS using GC-EI-MS: intra- and inter-batch precision (expressed as relative standard deviation) and accuracy (calculated as the percentage of the nominal concentration) determined at low, medium, and high GHB concentrations in DBS

Nominal concentration ($\mu\text{g/ml}$)	Found concentration ($\mu\text{g/ml}$)	Intra-batch precision (%RSD, $n=5$)	Inter-batch precision (%RSD, $n=6$)	Accuracy (% , $n=6$)
2	1.93	4.5	18.6 ^a	96
10	9.49	6.2	12.9	95
100	108.87	8.1	3.0	109

^a Negatively influenced by one value, which was statistically just not an outlier

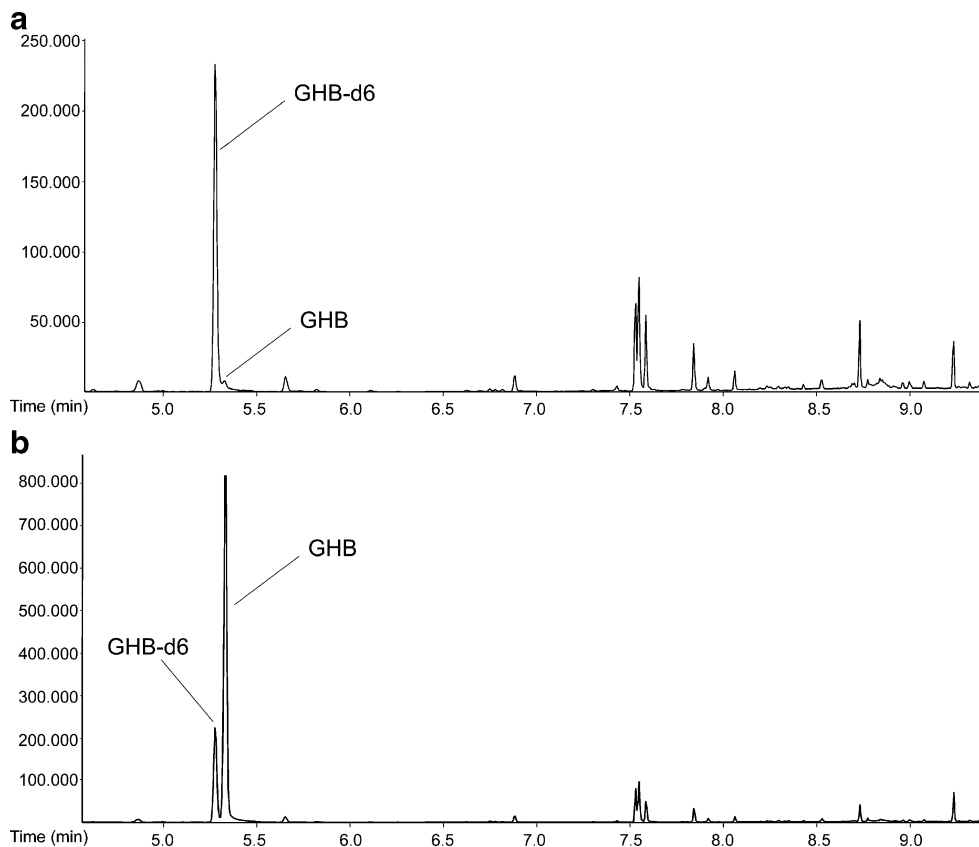
Table 3 Stability data of GHB in DBS, stored at room temperature up to 7 days, at 4 °C up to 24 h, and at -20 °C up to 14 days, presented as the percentage of the results obtained when analyzed after 4 h of drying, which is the recommended drying time according to the manufacturer

Room temperature			4 °C			-20 °C		
Time	Low	High	Time	Low	High	Time	Low	High
	(n=3), mean ± SD			(n=3), mean ± SD			(n=3), mean ± SD	
2 h	98.2±5.8	101.0±12.0	24 h	97.2±6.8	106.1±13.8	24 h	94.1±3.9	101.2±9.2
8 h	95.2±7.1	107.0±9.2				7 days	99.1±5.4	104.4±3.5
12 h	96.4±4.0	107.3±10.8				14 days	99.8±7.2	112.6±5.1
24 h	95.1±4.6	105.4±5.2						
48 h	98.1±7.7	102.8±2.9						
7 days	95.5±2.6	101.9±6.3						

analogues of GHB, and certain club and date rape drugs, with no interferences being observed. Not unexpectedly, the presence of a high concentration of GBL in the sample resulted in an increase of the GHB signal, likely due to conversion during sample preparation. The interconversion between GBL and GHB is well known and has extensively been investigated. It can be influenced by pH, temperature, and time [28, 50–52]. Upon spiking increasing concentrations of GBL to whole blood, followed by analyzing the resulting DBS, we saw an approximate 10% conversion of GBL to GHB, independent of the GBL concentration. This result is consistent with that reported by Sabucedo and

Furton [27] who found a 6.5% conversion. Because upon ingestion GBL is metabolized to GHB by serum lactonases within minutes in humans, normally no or only minimal amounts of GBL will be present in the sample. However, possible saturation of serum lactonases following ingestion of larger amounts of GBL has been reported, although this still remains unclear [24]. Anyway, cases in which GBL ingestion has occurred will likely readily have high GHB blood levels, and although the ±10% conversion of GBL to GHB may somewhat falsely elevate the quantitative result of GHB, this will likely have no relevance, neither in the forensic context nor in the clinical setting [24, 53].

Fig. 2 Chromatograms obtained when analyzing the derivatized extracts of DBS from two possible GBL users with GC-EI-MS in the SIM mode with a GHB concentration <1 µg/ml in the first sample (a) and a GHB concentration of 44.4 µg/ml in the second sample (b)



Stability

The stability of GHB in DBS was thoroughly investigated as previous studies reported an increase in GHB concentration during the preservation of blood samples withdrawn on certain anticoagulants [35, 42]. Therefore, to investigate the short-term stability of GHB in DBS samples on Whatman 903 filter paper, we evaluated low and high concentration levels ($n=3$) at different preservation temperatures (room temperature, 4 °C, and -20 °C). Table 3 shows that GHB in DBS at both low and high concentration levels is stable when stored for at least 1 week at room temperature, 24 h at 4 °C, and 14 days at -20 °C, as the mean percentages were within the predefined 15% limits. Based on these results, the collection of blood containing GHB on filter paper may result in a better storage manner of blood samples. Further examination is recommended to evaluate whether DBS are indeed an alternative and reliable way of routinely storing suspicious blood samples over a longer period of time. Furthermore, although in this study the DBS were dried for a minimum of 4 h at room temperature before analysis, in compliance with the manufacturer's recommendations, a shorter drying time of 2 h is also possible if needed.

Re-injection of processed samples after a waiting period of 24 h to verify autosampler stability of the derivatized extracts proved to be no problem as nearly no differences in peak area ratio were seen (RSD<2.1%). Also, the stability of processed samples stored for 1 week at -20 °C was acceptable (RSD<2.2%). Stock solutions were stable for a minimum of 14 days preserved at -20 °C and after three freeze-thaw cycles.

Application

Besides the screening and subsequent confirmation of positive findings with GC-MS and HPLC-DAD of blood and urine samples, also DBS prepared from the blood samples of two young men suspected of drug intake were analyzed with the described method. Results for the routine investigation of a blood sample of one young man led to the confirmation of the presence of alcohol (1.42‰), cocaine (benzoylecgonine), cannabinoids, MDMA, and ketamine. By analyzing the DBS, a GHB concentration of <1 µg/ml was found (Fig. 2a). In the blood of the other young man, alcohol (0.73‰), cannabinoids, and MDMA were present. The latter young man was also positive for GHB abuse as a 44.4 µg/ml GHB concentration was found by analyzing the DBS (Fig. 2b). These positive and negative results were confirmed by analyzing the whole blood samples for GHB with our previously published method [26], suggesting that our newly developed method may be applicable in routine samples in a toxicological laboratory for screening purposes, as well as for the confirmation of the presence of

GHB in whole blood samples. Interesting to note in this respect is that analysis of these DBS 5 months later (storage at -20 °C) yielded similar results.

Conclusion

A sensitive and accurate GC-MS method was developed for the determination of GHB in whole blood samples spot on Whatman 903 filter paper. The DBS sample procedure has advantages over the conventional blood collection, such as ease of handling and no specific storage requirements. Especially in the case of GHB, where an increase in GHB concentrations during the preservation of whole blood cannot be excluded, flexibility in storage conditions is of interest. Furthermore, as the detection window is limited, it is important that samples are obtained as early as possible after ingestion. By facilitating this, DBS may consequently represent an alternative in forensic and clinical cases where there is a suspicion of illicit use of GHB, in the case of DUID or when a DFSA is presumed.

Our LLOQ of 2 µg/ml is well below the proposed cutoff levels of 4 and 5 µg/ml for blood samples, so this method provides enough sensitivity to distinguish between endogenous and exogenously administered GHB, which is of major concern for the toxicological interpretation of clinical and forensic samples.

Finally, our approach of direct derivatization "on spot" may also be suitable for the determination of other compounds which impose extraction problems.

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