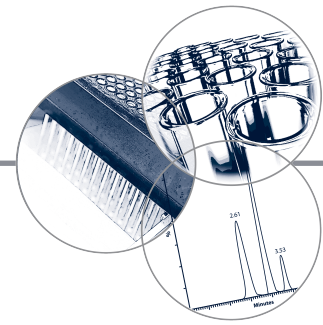
RESEARCH ARTICLE
SPECIAL FOCUS: DRIED BLOOD SPOTS





# Dried blood spot punches for confirmation of suspected γ-hydroxybutyric acid intoxications: validation of an optimized GC–MS procedure

**Background**: γ-hydroxybutyric acid (GHB), notorious as a club- and date-rape drug, was quantified in dried blood spots (DBS) by punching out a disc, followed by 'on-spot' derivatization and analysis by GC–MS. **Results**: A homogenous distribution in DBS was demonstrated and accurate results were obtained when analyzing a disc punched out from a 20–35 µl spot, regardless the hematocrit of the blood sample. Validation based on US FDA and European Medicines Agency guidelines was performed, with a calibration range covering 2–100 µg/ml. **Conclusion**: A sensitive GC–MS method for GHB analysis in DBS was successfully optimized and validated. The successful analysis of DBS collected from GHB abusers suggests the routine applicability of the DBS sampling technique for GHB analysis in toxicological cases.

The short chain fatty acid γ-hydroxybutyric acid (**GHB**) was synthesized in the early sixties as a structural analogue of γ-aminobutyric acid and also occurs naturally in blood, urine and peripheral and brain tissue [1,2]. Although the function of endogenous GHB has not completely been revealed yet, evidence suggests that it may act as a neuromodulator or neurotransmitter [2]. As a legal substance (sodium oxybate), GHB has a role as an anesthetic agent, in the treatment of narcolepsy with cataplexy and in alcohol and opiate withdrawal. In addition, it has also been sold as a substance of nutritional supplements to induce sleep and increase muscle mass. Currently, illegal GHB (liquid ecstasy) as well as its precursors, γ-butyrolacton and 1,4-butanediol, are popular as club drugs and appear occasionally in drug-facilitated sexual assaults [3]. In those toxicological cases, the interpretation of a positive analytical result is a real challenge, owing to its endogenous presence and reported *in vitro* production [4,5]. Therefore, **cut-off levels** have been proposed by several authors and these are currently set at 4 or 5 µg/ml for blood (serum) samples [2]. In addition, the detection window is very limited as GHB is rapidly metabolized and eliminated after oral ingestion (**plasma half-life** <1 h), so blood samples must be taken within 6 h after ingestion [6]. Consequently, a sampling delay may result in blood levels below the established cut-off level, no longer resulting in a positive case [7].

Blood sample collection may be facilitated by using **dried blood spot (DBS) sampling**. A DBS is capillary whole blood obtained by a finger or heel prick and collected on a filter paper card. Advantages over a venipuncture are the easy and rapid way to collect a representative sample and the less specific sample transport and storage requirements [8]. Previously, DBS sampling has generally been used for newborn screening, however, more recently, this alternative sampling strategy is increasingly gaining interest in the context of therapeutic drug monitoring and (pre-)clinical studies, as well as in toxicology [9,10]. We recently reported on the development and validation of a new procedure for GHB determination in DBS, using **'on-spot' derivatization** and **GC–MS** [11]. Similarly, other drugs of forensic interest have been determined in DBS, such as 3,4-methylenedioxymetamphetamine, morphine and 6-acetylmorphine or cocaine [12–14].

To obtain a DBS on filter paper card, a drop of blood can be spotted directly on the filter paper or with the aid of a precision capillary [8]. In our previous study, we used the second sample collection technique, and spotted a drop of blood with a fixed volume onto the filter paper card, followed by analysis of the complete DBS [11]. However, as correct sampling in this case ideally requires the presence of trained staff and in routine practice, it is more convenient to collect the drop of blood directly on the filter paper card, we therefore modified our procedure accordingly. As we did not wish this simplification to be at the expense of sensitivity (LLOQ of 2 µg/ml), we readjusted several sample pretreatment steps.

**Ann-Sofie Ingels[1], Peter De Paepe[2], Kurt Anseeuw[3], Diederik Van Sassenbroeck[4], Hugo Neels[5,6], Willy Lambert[1] & Christophe Stove[†1]**

[1]Laboratory of Toxicology, Faculty of Pharmaceutical Sciences, Ghent University, Harelbekestraat 72, B-9000 Ghent, Belgium
[2]Department of Emergency Medicine, Ghent University Hospital, De Pintelaan 185, B-9000 Ghent, Belgium
[3]Emergency Department, ZNA Stuivenberg, Lange Beeldekensstraat 267, B-2060 Antwerp, Belgium
[4]Emergency Care Department, AZ Maria Middelares, Kortrijksesteenweg 1026, B-9000 Ghent, Belgium
[5]Toxicological Center, Department of Pharmaceutical Sciences, University of Antwerp, Universiteitsplein 1, B-2610 Antwerp, Belgium
[6]Laboratory of Toxicology, ZNA Stuivenberg, Lange Beeldekensstraat 267, B-2060 Antwerp, Belgium
[†]Author for correspondence:
Tel.: +32 9264 8136
Fax: +32 9264 8183
E-mail: christophe.stove@ugent.be

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**Key Terms**

**GHB:** GHB or γ-hydroxybutyric acid is an endogenous short chain fatty acid. Although GHB administration has clinical use in selected cases, it is primarily known for its misuse as a club or date rape drug.

**Cut-off level for GHB:** Arbitrarily set decision limit allowing differentiation between endogenously present and exogenously administered GHB.

**Plasma half-life:** The time needed to reduce an original drug concentration in blood plasma by 50%.

**Dried blood spot sampling:** The process of collecting drops of blood on a filter paper card, followed by drying.

**'On spot' derivatization:** Procedure in which derivatization reagents are added directly on the dried spot, no longer requiring an extra drying step or an extraction solvent.

**GC–MS:** Gas chromatography, coupled to mass spectrometry; a commonly available analytical technique in toxicological laboratories used for determination of drugs and metabolites in biological matrices.

**Blood sample properties:** When punching out part of a DBS, no longer a fixed blood volume is analyzed, but a fixed area (disc diameter); therefore, blood sample properties, such as hematocrit, can influence the analytical result, requiring the investigation of additional parameters (influence of hematocrit, volume spotted, site of punching) during method development and validation.

Furthermore, the analysis of DBS punches rather than of complete DBS requires the evaluation of the impact of various **blood sample properties** [10]. In this study, the influence of the punch localization, the volume spotted on the filter paper card and of the hematocrit value (Ht) was evaluated in terms of precision and accuracy of the GHB concentration, measured in DBS samples [10]. Following method validation including the generally accepted parameters for bioanalytical methods, we demonstrated applicability by analyzing DBS collected from patients with a suspected GHB-intoxication at the emergency department. The results obtained from capillary sampling and those obtained by conventional blood collection (venipuncture) were compared in order to evaluate the DBS sampling technique.

## Experimental

### ■ Reagents

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

Stock and working solutions to prepare calibration solutions of GHB were prepared by dissolving 10 mg of the base in 1 ml methanol, followed by appropriate dilution to obtain 1 and 0.1 mg base/ml. To prepare QCs, a second independent stock solution was used. For the IS, a 25 µg/ml methanolic solution of the deuterated base was prepared by appropriate dilution of the commercially available stock solution, 1 mg Na-GHB-d6/ml, in methanol. All solutions were stored at -20°C.

### ■ DBS sampling

In the procedure to obtain DBS, the hand is first cleaned and then held down or warmed for a few minutes. With the help of an automatic lancet (Becton Dickinson reference number VAC366594, Franklin Lakes, USA), the fingertip is pricked. While the first drop of blood is wiped off with a sterile piece of cloth owing to the presence of tissue fluid, the following drops are collected on a Whatman 903 filter paper card (reference number 10334885, Dassel, Germany) with preprinted circles. The circle (8 mm diameter) must be entirely filled with blood and, although the blood is spot on just one side, both sides of the filter paper must be colored [8].

For method development and validation, we used venous whole blood from healthy non-user volunteers with endogenous GHB concentration below the established LLOQ, collected in ethylenediaminetetraacetic acid (EDTA) tubes and preserved for a maximum of 1 week at 4°C. No significant difference ($\alpha = 0.05$, 95% CI) was observed between the mean GHB concentration measured (nominal value 5 and 100 µg/ml; n = 5) when 25 µl of blood was either directly applied with a calibrated pipette or by allowing the drops to fall from the pipette tip onto the filter paper card. Thus, from the whole blood samples, 25 µl spots were applied with a calibrated pipette directly onto the Whatman filter paper. The resulting spots were dried for a minimum of 2 h at ambient temperature and subsequently analyzed or preserved in a zip-closure plastic bag with desiccant at room temperature until analysis.

### ■ Sample preparation & analytical procedure

Instead of using the whole DBS, only a 6 mm (diameter) disc (corresponding to ± 10 µl) was punched out from the center of a DBS. This influences the sample pretreatment procedure, thus each step from our previous procedure [11] was re-evaluated (data not shown). The most important adjustments included the addition of the IS (5 µl of a 25 µg/ml solution) to the punched disc, halving of the amount of derivatization reagents (TFAA and HFB-OH [2/1 v/v]), and halving of the amount of ethyl acetate to redissolve the dried derivatized sample. Supplementary Table 1 gives a detailed overview of all adjustments. Derivatized extracts were analyzed by GC–MS as described before, using the ratio of GHB to GHB-d6 (IS) for quantification [11]. The following ions were monitored using the selected ion monitoring mode: *m/z* 155, 183, 227 and 242 for derivatized GHB and *m/z* 161, 189, 231 and 245 for derivatized GHB-d6 (*m/z* 227 and 231 ions represent the quantifier ions, the other ions were selected as qualifier ions).

### ■ DBS method validation

As suggested by several authors, punching out a disc from a DBS has as a consequence that

the impact of additional parameters needs to be evaluated, such as the punch localization (at the periphery or central in the DBS), the influence of the volume spotted on the filter paper card and the Ht [10,15]. Furthermore, a partial validation was performed based on the US FDA and European Medicines Agency guidelines for the validation of bioanalytical methods [101,102]. Therefore, linearity, precision, accuracy, limits of detection and quantification and dilution integrity were evaluated. Also, long-term stability was determined [10]. Short-term stability, stability of stock solutions and selectivity of the method were evaluated during earlier validation experiments [11].

### Influence of the punch localization

Spots of 50 µl (n = 5) were prepared at both low and high GHB concentration levels in whole blood with low (0.38), intermediate (0.45) and high (0.50) Ht. The difference between the mean GHB concentrations obtained when analyzing discs punched out peripherally versus centrally was statistically evaluated using an independent sample t-test ($\alpha$ = 0.05; 95% CI) [16].

### Influence of the blood spot volume

Venous whole blood from healthy volunteers with low (0.38), intermediate (0.45) and high (0.50) Ht was spiked at both low and high GHB concentration levels, 5 and 100 µg/ml, respectively. Different volumes (20, 35 and 50 µl) were spotted (n = 5 or 6) onto filter paper card, the DBS were then dried and analyzed. To calculate accuracy, the obtained GHB concentrations, when using a calibration curve prepared in whole blood with intermediate Ht (0.45), were divided by the nominal value of 5 or 100 µg/ml and multiplied by 100%. The average % bias, which is the accuracy lowered with 100%, needed to be within ±15%, while the within-volume precision needed to be <15% relative standard deviation (RSD), calculated by dividing the standard deviation (SD) by the mean ratio of GHB to GHB-d6 and multiplying by 100% [15].

### Influence of the hematocrit

To investigate the effect of increasing Ht on the GHB concentration, both low and high GHB concentration solutions were prepared in six whole blood samples with increasing Ht (0.34, 0.39, 0.44, 0.46, 0.51 and 0.56) and 25 µl spots were made (n = 5). Therefore, we started from a whole blood sample, and after centrifugation, plasma was added or withdrawn to obtain whole blood samples with increasing Ht. The DBS were then analyzed as described above, with normalization of the results to those obtained for the sample with Ht of 0.44, as this was the theoretical average value of our patient population, including healthy women and men, with Ht reference ranges of 0.37–0.47 and 0.41–0.51, respectively [17].

### Validation

To obtain calibration data, on 4 non-consecutive days fresh calibration solutions were prepared in venous whole blood with intermediate Ht (0.45) and the resulting DBS were analyzed in duplicate. To ensure the independency of the result to the blood matrix properties, QC samples (2, 10 and 100 µg/ml) were prepared in venous whole blood samples with low (0.38), intermediate (0.45) and high (0.50) Ht values, obtained from different individuals [10]. For each day, a six-point calibration curve was constructed by plotting the ratio (mean of the duplicates) of the area of GHB to GHB-d6 in function of the concentration (2, 5, 10, 25, 50 and 100 µg/ml). The resulting data were statistically evaluated by performing weighting if necessary. Therefore, the sum % relative error (RE) was calculated and plotted against 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 [18].

Intra-batch precision was evaluated by analyzing six replicates of the QC solutions prepared in blood with intermediate Ht on the same day. The RSD was calculated as described above and needed to be <20% at the LLOQ and <15% at the other levels. Inter-batch precision was evaluated by analyzing the QC samples on 4 separate days in duplicate. The RSD was calculated using one-way ANOVA, as recently described by Wille *et al.,* and needed to be <20% at LLOQ and <15% at the other levels [19]. Accuracy needed to be within 85 and 115% of the nominal value, and within 80 and 120% at the LLOQ level.

To evaluate sensitivity, the LOD was estimated as the minimum GHB concentration with a signal/noise (S/N) ≥3. Furthermore, the LLOQ was defined as the lowest GHB concentration still measured with % RSD <20% and accuracy between 80 and 120%.

The possibility to dilute the final derivatized extract of samples with a GHB concentration

higher than that of the highest point of the calibration curve (100 µg/ml) was assessed by spiking venous whole blood with low (0.38), intermediate (0.45) and high (0.50) Ht at 200 µg/ml and 25 µl spots were made (3 days; n = 2). The spots were analyzed as described above, with 10 µl of the final derivatized extract diluted to 100 µl with ethyl acetate (as a result, the derivatized IS is also diluted tenfold). The mean GHB concentration was back-calculated by using the daily calibration curve and corrected for by the dilution factor. Inter-batch precision (% RSD) and accuracy were evaluated as described above and needed to be <15% and within 85 and 115%, respectively.

Finally, long-term stability at room temperature was investigated by analyzing DBS (n = 6) at both low and high GHB concentration levels (5 and 100 µg/ml) at time point zero, after 14, 48 and 148 days of storage. The mean concentration measured must be within ±15% of the nominal concentration, when using a freshly prepared calibration curve.

### ■ Determination of GHB in DBS collected at the emergency department

Patients transported to the emergency room of the cooperating hospitals (Ghent and Antwerp, Belgium) with moderate-to-severe loss of consciousness and/or with indications of GHB-ingestion were included in this study (approved by the local medical ethical boards). A venipuncture was performed (EDTA used as anticoagulant) and within 10 min capillary DBS were obtained as described above, in order to compare the GHB concentration in the venous and capillary whole blood sample [15]. Within 30 min after collection of the venous whole blood sample, DBS were prepared (so-called venous DBS) by applying 25 µl onto the filter paper card with a calibrated pipette. The collected DBS were left to dry for a minimum of 2 h at room temperature and were then placed in a zip-closure plastic bag with desiccant until analysis, while the venous whole blood samples were stored at 4°C until analysis. The venous whole blood samples were analyzed in accordance with the routine procedure for toxicological analysis, while the DBS were analyzed as described above in order to confirm a possible GHB-intoxication. If the GHB concentration was found to be above the highest calibration level, the derivatized extract was diluted as described. In addition, an aliquot of a GHB-positive venous whole blood sample was analyzed according to the procedure of Van hee *et al.* [20]. In brief, 20 µl of the whole blood sample was directly derivatized to obtain the di-trimethylsilyl derivative of GHB, which was analyzed by GC–MS in the selected ion monitoring mode.

The percentage difference between the various GHB measurements was calculated from the following concentration ratios: [venous DBS]/[capillary DBS] and [venous whole blood]/[venous DBS]. These respective ratios were used to evaluate whether there were consistent differences in GHB concentrations between, on the one hand, DBS obtained from capillary versus venous blood and, on the other, between venous blood analyzed as such or after having prepared DBS from it. Finally, the measured GHB concentrations obtained by analyzing venous whole blood versus capillary DBS were compared based on the ratio: [venous whole blood]/[capillary DBS].

## Results & discussion

Following DBS collection and drying (for a minimum of 2 h at room temperature), a 6 mm (diameter) disc was punched out. After applying the IS, 'on-spot' derivatization was performed with a mixture of TFAA and HFB-OH (50 µl, 2/1 by volume) at 60°C for 10 min. The derivatized sample was then centrifuged, dried under a gentle stream of nitrogen and subsequently redissolved in 100 µl ethyl acetate. Following brief sonication and centrifugation, 1 µl of the derivatized extract was analyzed by GC–MS. Besides modification of the sample preparation, the impact of additional parameters was investigated. Finally, the procedure was validated and applicability was demonstrated at the emergency department of cooperating hospitals.

### ■ DBS method validation

#### Influence of the punch localization

Several publications have pointed out that the site of punching may have an effect on the measured concentration. This has been shown for both macromolecules (proteins) as well as for small molecules, with higher concentrations observed at the peripheral or at the central punching site depending on the molecule under investigation. This effect, which is also influenced by the Ht, is likely owing to chromatographic effects, which are determined by the interaction of the compound with both the paper and the blood [21–23]. To investigate whether the site of punching out a disc from a

DBS influences the result of our analyses, discs punched out peripherally and centrally were analyzed. Irrespective of the Ht, no significant difference between the mean GHB concentrations at a confidence level of 95% was observed, demonstrating a homogenous GHB distribution in DBS [16].

### Influence of the blood spot volume

The influence of the volume spotted on the measured analyte concentration was evaluated by replicate analysis of discs punched out in the center of DBS with different blood volumes. Figure 1 summarizes the results and shows that the average % bias was overall within the predefined acceptance limits of ±15%, except for the analysis of discs punched out from 50 µl spots (5 µg/ml GHB) at the low and high Ht. The latter is probably due to an overload of the filter paper negatively influencing the spread and homogenous distribution of the blood drop. The within-volume precision (% RSD) was overall <15%. Therefore, based upon our results, the best blood volume spotted was between 20 and 35 µl, regardless of the Ht of the blood sample. This is also the volume required for filling the preprinted circles in the case of DBS from patients (8 mm diameter, containing ±20 µl).

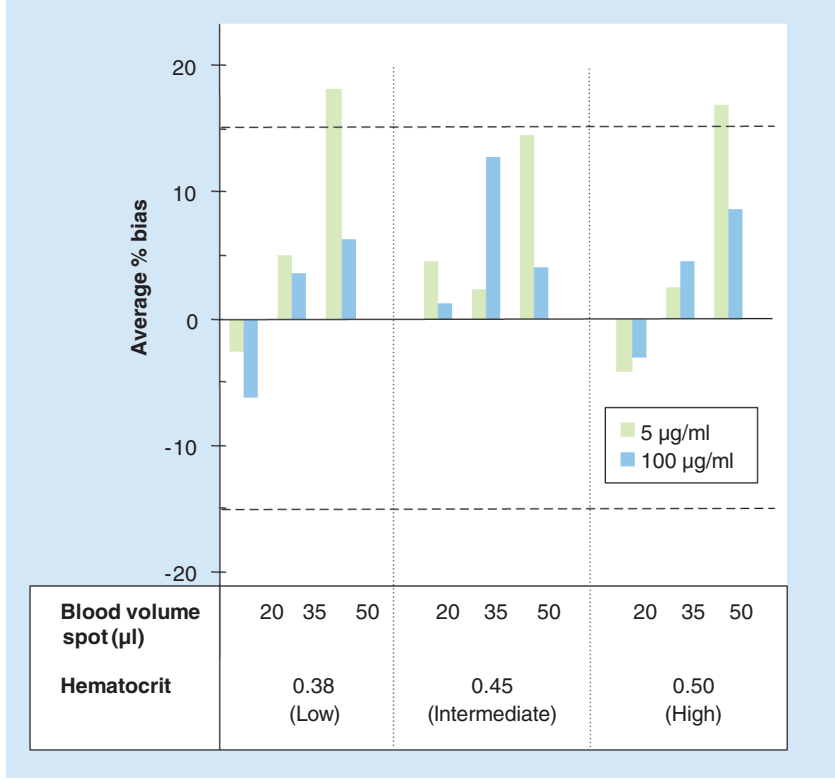


**Figure 1. Average % bias versus blood volume spotted for the determination of γ-hydroxybutyric acid in dried blood spots.** The dried blood spots (n = 5 or 6, at low and high nominal value) were analyzed using 'on-spot' derivatization and GC–MS, operating in selected ion monitoring mode. Dotted lines indicate the ±15% (bias) limits.

### Influence of the hematocrit

Although not unequivocally demonstrated, an equal distribution of GHB between plasma/serum and blood is assumed, which, similar to ethanol, is expected to result in a concentration ratio of blood-to-plasma serum of about 0.87 (this figure being slightly lower than 1 owing to the solid constituents of blood) [2,24]. As this implies an even partitioning between plasma/serum and erythrocytes, no effect of the Ht on the GHB concentration in blood per se is expected [25]. Still, the influence of the Ht requires special attention, especially in the analysis of DBS, as the Ht is directly proportional to the blood viscosity, affecting flux and diffusion of the blood, which is spotted on the filter paper card. The Ht values in healthy women and men range from 0.37 to 0.47 and from 0.41 to 0.51, respectively [17]. Using a calibration curve obtained by analyzing DBS prepared with blood with intermediate Ht (0.45), we determined the GHB concentration (low and high nominal value) in DBS from whole blood solutions with increasing Ht. The results are summarized in Table 1, presenting the % deviation from the normalized sample with average Ht [17]. Overall, we observed little or negligible influence in the Ht range of 0.39 to 0.51; covering the expected range of Ht in our patient population. However, analysis of DBS prepared from whole blood with Ht deviating from the reference range may no longer result in accurate measurements. Therefore, based upon this experiment and in agreement with other reports, for quantification

**Table 1. Influence of hematocrit on the γ-hydroxybutyric acid concentration measured in dried blood spot samples using GC–MS in selected ion monitoring mode.**

| Hematocrit | Low GHB concentration (5 µg/ml; n = 5) | High GHB concentration (100 µg/ml; n = 5) |
|---|---|---|
| 0.34 | -15.0 | -5.17 |
| 0.39 | -3.45 | -2.73 |
| 0.44 | Normalized | Normalized |
| 0.46 | -2.32 | 3.85 |
| 0.51 | -0.910 | 1.18 |
| 0.56 | 11.1 | 10.9 |

*Values indicate the % deviation from the γ-hydroxybutyric acid concentration obtained for the sample with a hematocrit of 0.44, which was used for normalization, given the reference interval of 0.37 to 0.51 for healthy women and men, respectively.*
*GHB: γ-hydroxybutyric acid.*

**Table 2. Calibration and sensitivity data for the determination of γ-hydroxybutyric acid in dried blood spots using GC–MS in selected ion monitoring mode.**

| Slope, mean ± SD (95% CI) | Intercept, mean ± SD (95% CI) | $R^2$ | Weighting factor | Range (µg/ml) | LOD (µg/ml) | LLOQ (µg/ml) |
|---|---|---|---|---|---|---|
| 0.044 ± 0.003 (0.040–0.047) | 0.000 ± 0.007 (-0.007–0.007) | 0.999 | $1/x^2$ | 2–100 | 1 | 2 |

*n* = 4.

purposes, it is recommended to prepare calibration and QC samples in whole blood with a Ht within the reference range and most preferably with an intermediate Ht, to minimize its effect on accuracy [8,10].

### Validation

The obtained calibration data were statistically evaluated as described in the experimental section and a weighting factor of $1/x^2$ was applied. The resulting calibration and sensitivity data are summarized in Table 2. Figure 2 shows a representative chromatogram for the LLOQ sample (2 µg/ml). Linearity was demonstrated within the working range using Fisher's test. As shown in Table 3, precision and accuracy were within the predefined acceptance limits (<15% RSD and bias). Results of the dilution experiment are summarized in Table 4. Precision and accuracy were acceptable, thus the derivatized extract of samples with a GHB concentration of more than 100 µg/ml can be diluted 1:10 with ethyl acetate prior to GC–MS analysis. Furthermore, DBS appeared to be stable when stored at room temperature in a zip-closure plastic bag with desiccant for at least 148 days, as the average calculated GHB concentration deviated less than 15% from the nominal value (Figure 3).

## ■ Determination of GHB in DBS collected at the emergency department

This study was conducted in order to evaluate the DBS sampling technique in a real-life setting, as well as to make a first comparison between the GHB concentrations measured in venous versus capillary whole blood. In total, 14 patients (between 18 and 35 years old, 13 men and one woman) were included, of whom blood sample analysis confirmed seven suspected GHB intoxications. Consistent with other reports describing GHB abuse in the context of multidrug use, we found GHB to be combined in all cases with other drugs such as alcohol, cocaine and cannabinoids [26]. We previously demonstrated that these drugs do not interfere with GC–MS determination [11]. The measured GHB concentrations in the collected DBS and whole blood are summarized in

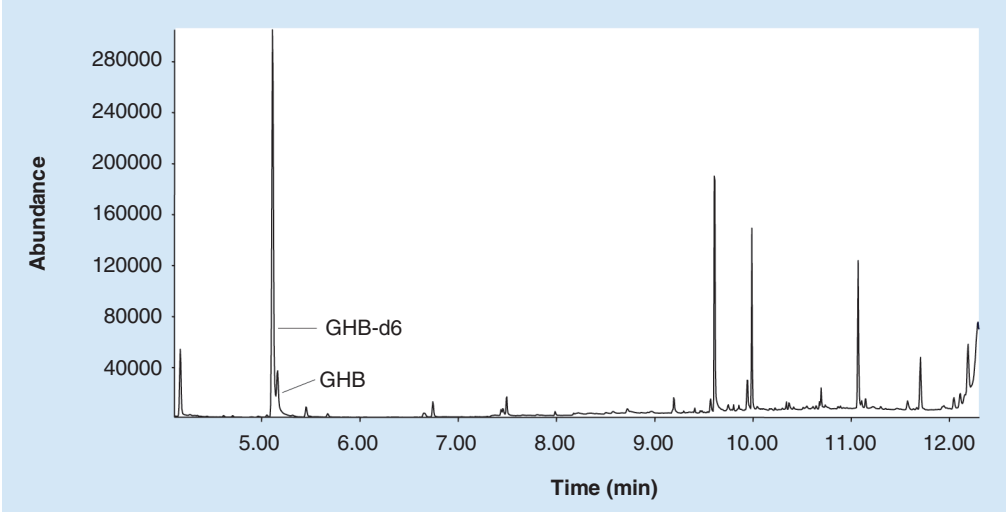


**Figure 2. Representative chromatogram obtained after analysis of a 6 mm disc punched out from a dried blood spot prepared from blood spiked with γ-hydroxybutyric acid at 2 µg/ml (LLOQ).** 5 µl of a 25 µg/ml internal standard solution was added to the punch before derivatization and analysis with GC–MS operating in selected ion monitoring mode.
GHB: γ-hydroxybutyric acid.

**Table 3. Inter- (n = 4; in duplicate) and intra- (n = 6) batch precision and accuracy data for the QC of γ-hydroxybutyric acid determination in dried blood spot samples prepared in whole blood with the indicated hematocrit value.**

| Nominal GHB concentration (µg/ml) | GHB concentration measured (µg/ml) | | | Precision (% RSD) | | | Accuracy (% nominal concentration) | | |
|---|---|---|---|---|---|---|---|---|---|
| | Low (0.38) | Int. (0.45) | High (0.50) | Low (0.38) | Int. (0.45) | High (0.50) | Low (0.38) | Int. (0.45) | High (0.50) |
| ***Interbatch*** | | | | | | | | | |
| 2 | 2.00 | 2.05 | 2.04 | 6.46 | 13.1 | 10.1† | 99.8 | 103 | 102 |
| 10 | 9.55 | 9.80 | 9.77 | 14.2 | 7.17† | 7.88 | 95.5 | 98.0 | 97.7 |
| 100 | 112 | 106 | 109 | 15.0 | 12.0 | 8.50 | 112 | 106 | 109 |
| ***Intrabatch*** | | | | | | | | | |
| 2 | | 1.96 | | | 4.93 | | | 97.9 | |
| 10 | | 9.03 | | | 6.13 | | | 90.3 | |
| 100 | | 101 | | | 4.10 | | | 101 | |

*Intrabatch data were obtained from blood with intermediate hematocrit.*
*†For 1 day of calibration, single analysis was performed of these QCs because of sample loss during sample preparation.*
*GHB: γ-hydroxybutyric acid; RSD: Relative standard deviation.*

Table 5. The mean difference seen between the measured GHB concentrations in the venous DBS versus capillary DBS was -8.7%. Although more paired sample analyses are recommended, these findings suggest that capillary DBS can be used for GHB determination. Figure 4 shows representative chromatograms obtained by GC–MS analysis of a derivatized extract of a capillary and venous DBS collected from the first GHB-positive patient (case number 1, measured GHB concentrations presented in Table 5). As stated by Li and Tse, for more than two-thirds of the samples the difference between a repeated measurement and the mean of the first and repeat measurement should be less than 20% [10]. This requirement was fulfilled when reanalyzing the capillary DBS after a minimum of one day (average of 8.9%; data not shown) [10]. Furthermore, to ensure that no consistent change in GHB concentration occurred during drying of the DBS, an aliquot of a GHB-positive sample was also analyzed using the procedure of Van hee *et al.* [20]. Overall, as shown in Table 5, analyzing a venous whole blood sample directly or after it has been spotted onto a filter paper card yielded similar analyte concentrations (mean difference of -5.8%). An average % difference of -13.2 was observed between the GHB concentrations measured in venous whole blood versus capillary DBS.

Concerning the sampling technique, collecting the drops of blood directly on the filter paper card was generally experienced as easy and quick. Also, thorough cleaning of the fingertips before sampling appeared to be very important to exclude contamination [8]. For example, we suspect the higher concentration found in the capillary DBS in case number 4 to be due to contamination. Nevertheless, the advantages of DBS sampling make this technique highly suitable for drug determination in a real-life setting, as it is less invasive than a venipuncture, the obtained DBS can be collected fast and only minimal sample volume is needed.

**Table 4. Results of the dilution experiment of samples with a γ-hydroxybutyric acid concentration exceeding the highest point (100 µg/ml) of the calibration curve (nominal value of 200 µg/ml).**

| Hematocrit | Back-calculated GHB concentration (µg/ml; n = 3; in duplicate) | Inter-batch precision (% relative standard deviation; n = 3; in duplicate) | Accuracy (% nominal concentration; n = 3; in duplicate) |
|---|---|---|---|
| Low (0.38) | 191 | 6.74 | 95.6 |
| Intermediate (0.45) | 210 | 6.36 | 105 |
| High (0.50) | 207 | 5.82 | 103 |

*The derivatized extract was diluted 1/10 with ethyl acetate prior to analysis by GC–MS. The measured GHB concentration was back-calculated, taking the dilution factor into account.*
*GHB: γ-hydroxybutyric acid.*

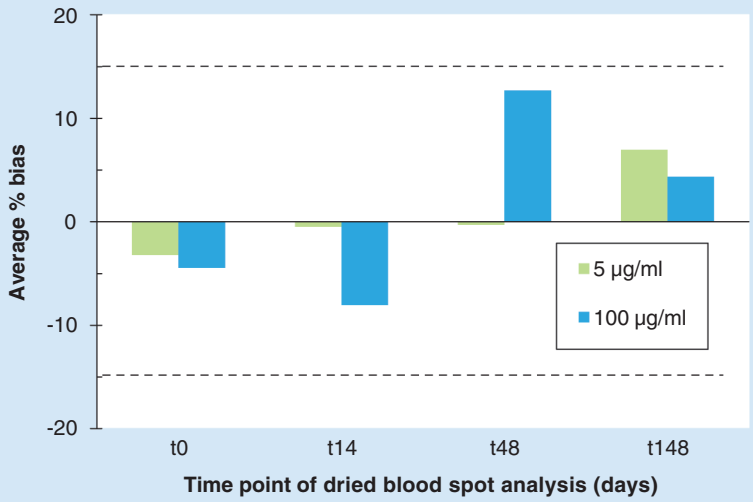


**Figure 3. Long-term stability of γ-hydroxybutyric acid in dried blood spots (n = 6, low and high γ-hydroxybutyric acid concentration level) stored at room temperature in a zip-closure plastic bag with desiccant up to 148 days.** The average % bias versus time point of dried blood spot analysis (days) is plotted and needed to be within the ±15% limits, indicated by the dotted lines. t0 refers to time point zero (dried blood spot analysis after 2 h of drying).

## Conclusion

A method for the determination of GHB in DBS, previously developed in our laboratory, was successfully adjusted in order to collect drops of blood directly on filter paper cards. Consequently, a fixed volume of blood was no longer analyzed, but only a 6 mm (diameter) disc punched out of the obtained DBS. This report includes the re-evaluation of the sample pre-treatment steps, in order to maintain a LLOQ of 2 µg/ml, as well as the evaluation of the impact of various blood sample properties and method validation. We applied this procedure in cooperating emergency departments by collecting and analyzing DBS from suspected GHB-users. Collecting the drops of blood directly on the filter paper card was experienced as a more convenient technique than the use of a precision capillary in a real-life setting. Moreover, GHB in DBS was found to be stable for at least 148 days when stored at room temperature in a zip-closure plastic bag with desiccant, and therefore overcomes the reported possibility of *in vitro* production by storage of whole blood samples at temperatures above 2–8°C [2]. To conclude, the collection and analysis of DBS may be a useful tool to confirm a suspected GHB ingestion, even outside a hospital environment, owing to the general advantages of this sampling technique.

## Future perspective

Although it is not expected that DBS will replace the conventional matrices such as whole blood, plasma, serum and urine in toxicological analysis [10], it can still be of interest to collect DBS because of the typical benefits associated with this sampling technique. Not only may the speed of sample collection be of interest (especially for compounds disappearing rapidly from the circulation, such as GHB), but also the fact that no specific staff, transport or storage conditions are required. This makes the use of DBS sampling an option that can be considered in drug-facilitated sexual assaults, driving under the influence of drugs (DUID), epidemiological studies [27], therapeutic drug monitoring, blood sampling of neonates, and young children or other patients with restricted venous access and for sampling at home. DBS have the advantage that many compounds remain stable, even when degradation or *in vitro* production has been observed in the conventional

**Table 5. γ-hydroxybutyric acid level (µg/ml) in paired venous and capillary dried blood spots and venous whole blood samples in seven patients presenting at the emergency department with a suspected γ-hydroxybutyric acid-intoxication.**

| GHB-positive case No. | Venous DBS (µg GHB/ml) | Capillary DBS (µg GHB/ml) | Venous WB (µg GHB/ml) |
|---|---|---|---|
| 1 | 81.4 | 78.8 | 88.5 |
| 2 | 170 | 150 | 173 |
| 3 | 153 | 169 | 154 |
| 4 | 56.3 | 91.7† | 44.0 |
| 5 | 118 | 142 | 126 |
| 6 | 107 | 116 | 90.5 |
| 7 | 127 | 132 | 97.0 |

*A DBS (single analysis) was analyzed using 'on-spot' derivatization and GC–MS, operating in selected ion monitoring mode, while venous whole blood (in duplicate) was analyzed according to the procedure of* Van hee *et al.* [20].
†*We suspect this higher value to be due to contamination of the fingertip.*
*DBS: Dried blood spot; GHB: γ-hydroxybutyric acid; WB: Whole blood.*

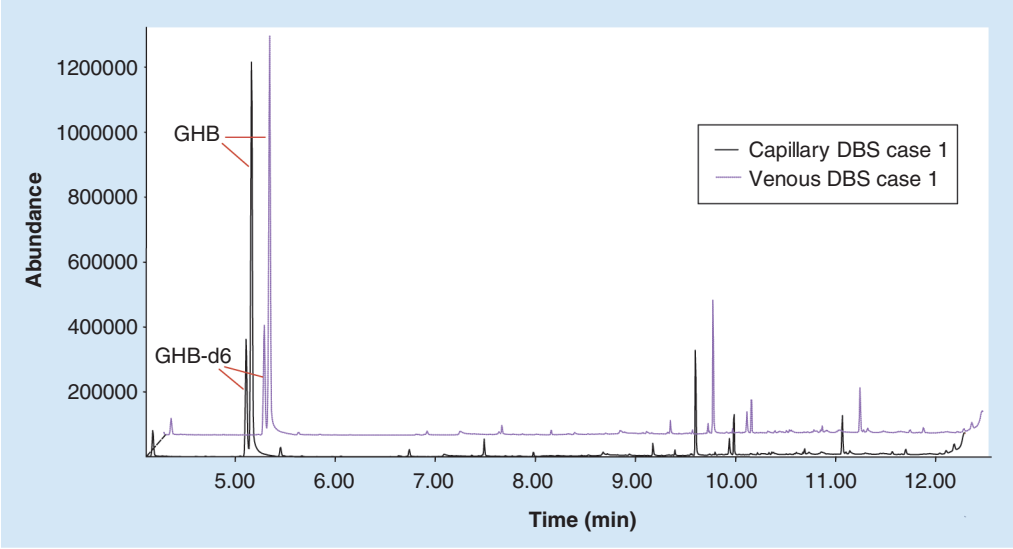


**Figure 4. Overlay of representative chromatograms obtained by analyzing the derivatized extract of a paired capillary and venous dried blood spot.** The DBS were collected from the first patient who tested positive for GHB use (GHB-positive case number 1 in Table 5) and were analyzed by using 'on-spot' derivatization and GC–MS, operating in selected ion monitoring mode. DBS: Dried blood spot; GHB: γ-hydroxybutyric acid.

matrices [28]. We do not consider any limitation with respect to the kind of molecules that can be determined using DBS analysis (from classical low-molecular-weight drugs to macromolecules, such as proteins), obviously taking into account the limits set by the sensitivity of the applied techniques.

On the other hand, the use of DBS as alternative sampling technique for the collection of a representative blood sample requires a substantial amount of preceding experiments demonstrating the similarity between the results measured in whole blood versus plasma, as well as versus DBS and the independency of the result from the blood matrix properties. Another limitation that might be experienced is the difficulty of performing re-analysis or multiple analyses [12]. This is only possible when spare sample is left; a possible solution might be the use of smaller discs with only 3 mm diameter, representing an even smaller amount of sample. Moreover, harmonized guidelines for the validation of DBS analysis methods should be established to investigate if a particular analytical method is suitable for DBS analysis of a study compound in routine practice.

Automatic procedures for DBS punching and extraction coupled to MS/MS are being developed to increase sample throughput [29,30]. In our opinion, GC–MS may also play a role in the determination of drugs in DBS, especially for molecules that impose extraction problems (which may be overcome by using 'on-spot' derivatization) and for small molecules, where the use of LC–MS/MS has little, if any, benefit with respect to sensitivity.

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## Financial & competing interests disclosure

*The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.*

*No writing assistance was utilized in the production of this manuscript.*

## Ethical conduct of research

*The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.*

## Executive summary

- By facilitating blood sampling, the dried blood spot (DBS) technique is an easy and rapid way to collect a representative blood sample.
- Accurate analysis of a disc punched out of a DBS requires the investigation of the influence of the punch localization, blood spot volume and hematocrit value.
- Sample work-up for the determination of γ-hydroxybutyric acid (GHB) in DBS is easy to perform, including 'on-spot' derivatization of the 6 mm punch, with a mixture of trifluoroacetic acid anhydride and heptafluorobutanol (2:1 v/v, for 10 min at 60°C).
- A GC–MS method was successfully validated: calibration was performed using an internal standard, GHB-d6, and acceptable sensitivity, precision, accuracy, dilution integrity and stability were obtained.
- Applicability was demonstrated by collecting DBS from patients presenting at the emergency department with a suspected GHB intoxication. Our findings support the possibility to use DBS for GHB determination.

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Supplementary file:

Overview of the sample collection and sample pre-treatment of our previously published method (method 1) and the newly developed method (method 2) for the determination of GHB in DBS with GC-MS operating in SIM mode

<table>
<tr><th>Method 1</th><th>Method 2</th></tr>
<tr><td colspan="2">Sample collection</td></tr>
<tr><td>50 µl capillary whole blood is spotted onto a Whatman 903 filter paper</td><td>A drop of blood is collected directly onto a Whatman 903 filter paper</td></tr>
<tr><td colspan="2">Sample pre-treatment</td></tr>
<tr><td>The complete DBS is excised<br><br>The IS is added (before excising the DBS)<br>10 µl of a 0.05-mg/ml methanolic solution<br><br>The DBS is left to dry for 15 min<br><br>100 µl derivatization reagents is added</td><td>A 6-mm punch is excised<br><br>The IS is added (after punching out)<br>5 µl of a 0.025-mg/ml methanolic solution<br><br>The punch is dried for 5 min under a gentle stream of nitrogen<br><br>50 µl derivatization reagents is added</td></tr>
<tr><td colspan="2">TFAA – HFB-OH (2:1, by volume) freshly prepared mixture<br>Sonication (2-5 min)<br>Derivatization at 60 °C for 10 min<br><br>The DBS is cooled down by centrifugation for 5 min at 4 °C<br><br>The sample is dried under a gentle stream of nitrogen at 25 °C</td></tr>
<tr><td>The sample is redissolved in 200 µl ethylacetate<br><br>Sonication for 5 min<br><br>Centrifugation 5 min 1.6 x 1000 g: 2x</td><td>The sample is redissolved in 100 µl ethylacetate<br><br>Sonication for 2 min<br><br>Centrifugation 5 min 1.6 x 1000 g</td></tr>
<tr><td colspan="2">Transfer of the supernatant to a vial</td></tr>
</table>