biblio.ugent.be

The UGent Institutional Repository is the electronic archiving and dissemination platform for all UGent research publications. Ghent University has implemented a mandate stipulating that all academic publications of UGent researchers should be deposited and archived in this repository. Except for items where current copyright restrictions apply, these papers are available in Open Access.

This item is the archived peer-reviewed author-version of:

Title: Dried blood spot punches for confirmation of suspected ϒ-hydrobutyric acid intoxications: validation of an optimized GC-MS procedure

Authors: Ingels Ann-Sofie, De Paepe Peter, Anseeuw Kurt, Van Sassenbroeck Diederik, Neels Hugo, Lambert Willy, Stove Christophe

In: Bioanalysis 3 (20), 2271-2281, 2011

To refer to or to cite this work, please use the citation to the published version:

Ingels Ann-Sofie, De Paepe Peter, Anseeuw Kurt, Van Sassenbroeck Diederik, Neels Hugo, Lambert Willy, Stove Christophe (2011). Dried blood spot punches for confirmation of suspected ϒ-hydrobutyric acid intoxications: validation of an optimized GC-MS procedure. Bioanalysis 3 (20), 2271-2281. DOI 10.4155/BIO.11.204

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

Ann-Sofie M.E. Ingels[1], Peter De Paepe[2], Kurt Anseeuw[3], Diederik K. Van Sassenbroeck[4], Hugo Neels[5,6], Willy E. Lambert[1], and Christophe P. 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 Centre, 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*

*Corresponding author:

Christophe P. Stove

Ghent University

Faculty of Pharmaceutical Sciences

Laboratory of Toxicology

Harelbekestraat 72

9000 Ghent

Belgium

E-mail: Christophe.Stove@UGent.be

tel.: +32 9 264 81 36

fax: +32 9 264 81 83

Abstract

Background: Gamma-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 gas chromatography coupled to mass spectrometry (GC-MS).

Results: A homogenous distribution in DBS was demonstrated, and accurate results were obtained when analyzing a disc punched out from a 20 to 35-µl spot, regardless the hematocrit of the blood sample. Validation based on FDA and EMA guidelines was performed, with a calibration range covering 2 to 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.

1. Introduction

The short chain fatty acid gamma-hydroxybutyric acid (GHB) was synthesized in the early sixties as a structural analogue of gamma-aminobutyric acid and occurs also 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 it may act as a neuromodulator or neurotransmitter [2]. As a legal substance (sodium oxybate), GHB has a role as an anaesthetic agent, in the treatment of narcolepsy with cataplexy and of 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 gamma-butyrolacton (GBL) and 1,4-butanediol are popular as club drugs and appear occasionally in drug-facilitated sexual assaults (DFSA) [3]. In those toxicological cases, the interpretation of a positive analytical result is a real challenge, because of its endogenous presence and the 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 as to a venipuncture are the easy and rapid way to collect a representative sample and the less specific sample transport and storage requirements [8]. Whereas DBS sampling has generally been used for newborn screening, 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 gas chromatography coupled to mass spectrometry (GC-MS) [11]. Also 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 a 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, so we spotted a drop with a fixed volume onto the filter paper card and analyzed 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 cards, we modified our procedure accordingly. As we did not wish this simplification to be at the expense of sensitivity (lower limit of quantification or LLOQ of 2 µg/ml), we re-adjusted several sample pre-treatment steps. Furthermore, the analysis of DBS punches rather than of complete DBS also requires the evaluation of the impact of various blood sample properties [10]. In this study, the influence of the punch localisation, of 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 bio-analytical methods, we demonstrated applicability by analyzing DBS collected from patients presenting at the emergency department with a suspected GHB-intoxication. The results obtained from capillary sampling and those obtained by conventional blood collection (venipuncture) were compared in order to evaluate the DBS sampling technique.

2. Experimental

2.1 Reagents

GHB (sodium salt, powder) and a 1-mg/ml solution in methanol of the internal standard (IS) GHB-d6 (sodium salt) 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 quality controls (QC's), 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 of 1-mg Na-GHB-d6/ml in methanol. All solutions were stored at -20 °C.

2.2 DBS sampling

In the procedure to obtain DBS, the hand is first cleaned and held down or warmed for a few minutes. With the help of an automatic lancet (Becton Dickinson ref n° VAC366594, Franklin Lakes, USA), the fingertip is pricked. While the first drop of blood is wiped off with a sterile piece of cloth because of the presence of tissue fluid, the following drops are collected on a Whatman 903 filter paper card (ref n° 10334885, Dassel, Germany) with pre-printed 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 coloured [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 EDTA tubes and preserved for maximum one week at 4 °C. No significant difference ($\alpha=0.05$, 95% confidence interval) 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. So 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 minimum 2 hours at ambient temperature and subsequently analyzed or preserved in a zip-closure plastic bag with desiccant at room temperature until analysis.

2.3 Sample preparation and analytical procedure

Instead of using the whole DBS, only a 6-mm (diameter) disc (corresponding to ± 10 µl) was punched out from the centre of a DBS. This influences the sample pre-treatment procedure, so each step from our previous procedure [11] was re-evaluated (data not shown). The most important adjustments included 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, by volume), and of ethyl acetate to redissolve the dried derivatized sample. The supplementary file 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 (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, the other ions were selected as qualifier ions).

2.4 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 localisation (at the periphery or central in the DBS), the influence of the volume spotted on the filter paper card and of the Ht [10, 15]. Furthermore, a partial validation was performed based on the FDA and EMA guidelines for the validation of bio-analytical 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].

2.4.1 Influence of the punch localisation

Fifty-µl spots (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 *vs.* centrally was statistically evaluated using an independent sample T-test (α=0.05, 95% confidence interval) [16].

2.4.2 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). Different volumes (20, 35 and 50 µl) were spotted (n=5 or 6) onto the filter paper card, the DBS were dried and subsequently 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].

2.4.3 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 analyzed as described above, and the sample with a Ht of 0.44 was normalized, as this is the theoretical average value of our patient population, including healthy women and men (Ht reference range of 0.37-0.47 and 0.41-0.51, respectively) [17].

2.4.4 Validation

To obtain the 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 6-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 the % RE versus concentration was plotted, 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 6 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 < 15 % and < 20 % at the LLOQ. 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 < 15 % and < 20 % at LLOQ [19]. Accuracy needed to be within 85 to 115 % of the nominal value and within 80 to 120 % at LLOQ level.

To evaluate sensitivity, the limit of detection (LOD) was estimated as the minimum GHB concentration with a signal-to-noise ratio equal to or larger than 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 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 and 10 µl of the final derivatized extract was diluted to 100 µl with ethyl acetate (as a result, also the derivatized internal standard is diluted 10-fold). The mean GHB concentration was back-calculated by using the daily calibration curve, and was corrected for by the dilution factor. Inter-batch precision (% RSD) and accuracy were evaluated and needed to be < 15 % and within 85-115 %, 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, and 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.

2.5 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 a GHB-ingestion were included in this study (approved by the local medical ethical boards). A venipuncture was performed (EDTA as anti-coagulant) and within 10 minutes 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 minutes 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 minimum 2 hours 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 of 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]. Briefly, 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 SIM mode.

The % 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 DBS obtained from capillary *vs.* venous blood and between venous blood analyzed as such or as DBS. Finally, the measured GHB concentrations obtained by analyzing venous whole blood *vs.* capillary DBS were compared based on the ratio [venous whole blood]/[capillary DBS].

3. Results and discussion

Following DBS collection and drying (for a minimum of 2 hours 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 the dried extract was 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.

3.1. DBS method validation

3.1.1 Influence of the punch localisation

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 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 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, this revealed no significant difference between the mean GHB concentrations at a confidence level of 95 %, demonstrating a homogenous GHB distribution in DBS [16].

3.1.2 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 centre of DBS with different blood volumes. Fig. 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 the homogenous distribution of the blood drop. The within-volume precision (% RSD) was overall < 15 %. So, 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 pre-printed circles in the case of DBS from patients (8-mm diameter, containing ± 20 µl).

3.1.3 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 or serum of about 0.87 (this figure being slightly lower than 1 because of 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]. However, 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 that 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 of 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 range from 0.39 to 0.51 Ht, covering the expected range of Ht in our patient population. Analysis of DBS prepared from whole blood with Ht deviating from the reference range, however, may no longer result in accurate measurements. Therefore, based upon this experiment and in agreement with other reports, for quantification 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, and this to minimize its effect on accuracy [8, 10].

3.1.4 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. Fig. 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, so the derivatized extract of samples with a GHB concentration of more than 100 µg/ml can be diluted 1 to 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 (Fig. 3).

3.2 Determination of GHB in DBS collected in the emergency room

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 *vs.* capillary whole blood. In total, 14 patients (between 18 and 35 years old, 13 men and 1 woman) were included, of whom blood sample analysis confirmed 7 suspected GHB intoxications. Consistent with other reports describing GHB abuse in the context of multi-drug 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 do not interfere with the GC-MS determination [11].

The measured GHB concentrations in the collected DBS and whole blood are summarized in Table 5. The mean difference seen between the measured GHB concentrations in the venous DBS *vs.* capillary DBS was -8.7 %. Although more paired sample analyses are recommended, these findings suggest that capillary DBS can be used for GHB determination. Fig. 4 shows representative chromatograms (overlay) obtained by GC-MS analysis of a derivatized extract of a capillary and venous DBS collected from the first GHB-positive patient (case n°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 %. This requirement was fulfilled when re-analyzing the capillary DBS after minimum 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 vs. 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 n° 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 little sample volume is needed.

4. Conclusion

A method for the determination of GHB in dried blood samples, previously developed in our laboratory, was successfully adjusted in order to collect drops of blood directly on filter paper cards. Consequently, no longer a fixed volume of blood was 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, 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 stored at room temperature in a zip-closure plastic bag with desiccant, so the DBS matrix overcomes the reported possibility of *in vitro* production by storage of whole blood samples at temperatures above 2 to 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, because of the general advantages coupled to this sampling technique.

5. Executive summary

- By facilitating blood sampling, the 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 localisation, of the blood spot volume, and of the Ht.
- Sample work-up is easy to perform including "on spot" derivatization of the 6-mm punch with a mixture of trifluoroacetic acid anhydride (TFAA) and heptafluorobutanol (HFB-OH) (2:1 by volume, 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.

6. Future perspective

Although it is not expected that DBS will replace the conventional matrices like whole blood, plasma, serum and urine in toxicological analysis [10], it can be of interest to collect DBS because of the typical benefits associated with this sampling technique. Not only the speed of sample collection may 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 DFSA or in driving under the influence of drugs (DUID), in epidemiological studies [27], in therapeutic drug monitoring, in blood sampling of neonates, young children or other patients with restricted venous access or 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 matrices [28]. We do not consider any limitation with respect to the kind of molecules that can be determined via DBS analysis (from classical low molecular weight drugs to macromolecules like 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 *vs.* plasma and *vs.* 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]. To our opinion, also GC-MS may 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.

Acknowledgements

The authors would like to thank GE Healthcare for kindly providing the Whatman 903 filter paper, the staff of the cooperating emergency units for their kind help with sampling and the volunteers who gave on a regular basis venous whole blood in order to conduct method development and validation.

References

[1] Andresen H, Stimpfl T, Sprys N, Schnitgerhans T and Muller A. Liquid ecstasy - a significant drug problem. *Dtsch. Arztebl. Int.* 105(36), 599-603 (2008).

[2] Andresen H, Sprys N, Schmoldt A, Mueller A and Iwersen-Bergmann S. Gamma-Hydroxybutyrate in Urine and Serum: Additional Data Supporting Current Cut-Off Recommendations. *Forensic Sci. Int* .200(1-3), 93-99 (2010).

[3] Carter LP, Pardi D, Gorsline J and Griffiths RR. Illicit gamma-hydroxybutyrate (GHB) and pharmaceutical sodium oxybate (Xyrem): differences in characteristics and misuse. *Drug Alcohol Depend.* 104(1-2), 1-10 (2009).

[4] Shima N, Miki A, Kamata T, Katagi M and Tsuchihashi H. Endogenous level and in vitro production of GHB in blood from healthy humans, and the interpretation of GHB levels detected in antemortem blood samples. *J. Health Sci.* 51(2), 147-154 (2005).

[5] Berankova K, Mutnanska K and Balikova M. Gamma-hydroxybutyric acid stability and formation in blood and urine. *Forensic Sci. Int.* 161(2-3), 158-162 (2006).

[6] Abanades S, Farre M, Segura M *et al.* Gamma-hydroxybutyrate (GHB) in humans: pharmacodynamics and pharmacokinetics. *Ann. N Y Acad. Sci.* 1074, 559-576 (2006).

[7] Jones AW, Holmgren A and Kugelberg FC. Driving under the influence of gamma-hydroxybutyrate (GHB). *Forensic Sci. Med. Pathol.* 4(4), 205-211 (2008).

[8] Edelbroek PM, Van Der Heijden J and Stolk LM. Dried blood spot methods in therapeutic drug monitoring: methods, assays, and pitfalls. *Ther. Drug Monit.* 31(3), 327-336 (2009).

[9] De Boer T, Wieling J, Meulman E *et al.* Application of dried blood spot sampling combined with LC-MS/MS for genotyping and phenotyping of CYP450 enzymes in healthy volunteers. *Biomed. Chromatogr.* Epub ahead of print DOI 10.1002/bcm.1580 (2011).

[10] Li W and Tse F. Dried blood spot sampling in combination with LC-MS/MS for quantitative analysis of small molecules. *Biomed. Chromatogr.* 24(1), 49-65 (2010).

[11] Ingels A, Lambert W and Stove C. Determination of gamma-hydroxybutyric acid in dried blood spots using a simple GC-MS method with direct "on spot" derivatization. *Anal. Bioanal. Chem.* 398(5), 2173-2182 (2010).

[12] Garcia Boy R, Henseler J, Mattern R and Skopp G. Determination of morphine and 6-acetylmorphine in blood with use of dried blood spots. *Ther. Drug Monit.* 30(6), 733-739 (2008).

[13] Mercolini L, Mandrioli R, Gerra G and Raggi MA. Analysis of cocaine and two metabolites in dried blood spots by liquid chromatography with fluorescence detection A novel test for cocaine and alcohol intake. *J. Chromatogr. A* 1217(46), 7242-7248 (2010).

[14] Jantos R, Veldstra JL, Mattern R, Brookhuis KA and Skopp G. Analysis of 3,4-Methylenedioxymetamphetamine: Whole Blood Versus Dried Blood Spots. *J. Anal. Tox.* 35(5), 269-273 (2011).

[15] Spooner N, Lad R and Barfield M. Dried Blood Spots as a Sample Collection Technique for the Determination of Pharmacokinetics in Clinical Studies: Considerations for the Validation of a Quantitative Bioanalytical Method. *Anal. Chem.* 81(4), 1557-1563 (2009).

[16] Kolocouri F, Dotsikas Y and Loukas YL. Dried plasma spots as an alternative sample collection technique for the quantitative LC-MS/MS determination of gabapentin. *Anal. Bioanal. Chem.* 398(3), 1339-1347 (2010).

[17] Wilhelm AJ, Den Burger JCG, Vos RM, Chahbouni A and Sinjewel A. Analysis of Cyclosporin a in Dried Blood Spots using Liquid Chromatography Tandem Mass Spectrometry. *J. Chromatogr. B* 877, 1595-1598 (2009).

[18] Almeida AM, Castel-Branco MM and Falcao AC. Linear regression for calibration lines revisited: weighting schemes for bioanalytical methods. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 774(2), 215-222 (2002).

[19] Wille SMR, Peters FT, Di Fazio V and Samyn N. Practical aspects concerning validation and quality control for forensic and clinical bioanalytical quantitative methods. *Accred. Quality Assur.* 16(6), 279-292 (2011).

[20] Van Hee P, Neels H, De Doncker M *et al.* Analysis of gamma-hydroxybutyric acid, DL-lactic acid, glycolic acid, ethylene glycol and other glycols in body fluids by a direct injection gas chromatography-mass spectrometry assay for wide use. *Clin. Chem. Lab. Med.* 42(11), 1341-1345 (2004).

[21] Schutt BS, Weber K, Elmlinger MW and Ranke MB. Measuring IGF-I, IGFBP-2 and IGFBP-3 from dried blood spots on filter paper is not only practical but also reliable. *Growth Horm. IGF Res.* 13(2-3), 75-80 (2003).

[22] Holub M, Tuschl K, Ratschmann R *et al.* Influence of hematocrit and localisation of punch in dried blood spots on levels of amino acids and acylcarnitines measured by tandem mass spectrometry. *Clin. Chim. Acta* 373(1-2), 27-31 (2006).

[23] Vu DH, Koster RA, Alffenaar JW, Brouwers JR and Uges DR. Determination of moxifloxacin in dried blood spots using LC-MS/MS and the impact of the hematocrit and blood volume. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 879(15-16), 1063-1070 (2011).

[24] Rainey PM. Relation between Serum and Whole-Blood Ethanol Concentrations. *Clin. Chem.* 39(11), 2288-2292 (1993).

[25] Emmons G and Rowland M. Pharmacokinetic considerations as to when to use dried blood spot sampling. *Bioanalysis* 2(11), 1791-1796 (2010).

[26] Elliott SP. Nonfatal instances of intoxication with gamma-hydroxybutyrate in the United Kingdom. *Ther. Drug Monit.* 26(4), 432-440 (2004).

[27] Wood DM, Nicolaou M and Dargan PI. Epidemiology of Recreational Drug Toxicity in a Nightclub Environment. *Substance Use & Misuse* 44(11), 1495-1502 (2009).

[28] D'arienzo CJ, Ji QC, Discenza L *et al.* DBS sampling can be used to stabilize prodrugs in drug discovery rodent studies without the addition of esterase inhibitors. *Bioanalysis* 2(8), 1415-1422 (2010).

[29] Abu-Rabie P and Spooner N. Direct Quantitative Bioanalysis of Drugs in Dried Blood Spot Samples Using a Thin-Layer Chromatography Mass Spectrometer Interface. *Anal. Chem.* 81(24), 10275-10284 (2009).

[30] Déglon J, Thomas A, Mangin P and Staub C. Direct analysis of dried blood spots coupled with mass spectrometry: concepts and biomedical applications. *Anal. Bioanal. Chem.* Epub ahead of print DOI 10.1007/s00216-011-5161-6 (2011).

[101] US FDA. Guidance for Industry: Bioanalytical method validation. Rockville, MD, USA: US Department of Health and Human services, FDA, Centre for Drug Evaluation and Research, 2001. www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070107

[102] European Medicines Agency (EMA). Draft Guideline on Validation of Bioanalytical Methods. London, UK. www.ema.europa.eu/pdfs/human/ewp/19221709en.pdf

Reference annotations

[2] * Interesting article about the proposed cut-off levels for GHB determination in urine and serum, with comparison to former propositions

[3] * This article clearly reviews the use and abuse of GHB

[8] * General overview of the use of DBS, the sampling technique, the analytical methods and the possible errors introduced by DBS collection

[10] ** Recent comprehensive overview on DBS method development and validation procedures

[11] * First application of "on spot" derivatization of DBS

Key terms

GHB:

GHB or gamma-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

PLASMA HALF-LIFE:

The time needed to reduce an original drug concentration in blood plasma by 50 %

CUT-OFF LEVEL for GHB:

Arbitrarily set decision limit allowing differentiation between endogenously present and exogenously administered GHB

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.

List of abbreviations

DBS	dried blood spots
DFSA	drug facilitated sexual assault
DUID	driving under the influence of drugs
CI	confidence interval
EDTA	ethylenediaminetetraacetic acid
EMA	European Medicines Agency
FDA	Food and Drug Administration
GBL	gamma-butyrolacton
GC-MS	gas chromatography coupled to mass spectrometry

GHB	gamma-hydroxybutyric acid
GHB-d6	deuterated gamma-hydroxybutyric acid
HFB-OH	heptafluorobutanol
Ht	hematocrit value
Int	intermediate
IS	internal standard
LC-MS/MS	liquid chromatography coupled to tandem mass spectrometry
LLOQ	lower limit of quantification
LOD	limit of detection
MS/MS	tandem mass spectrometry
QC	quality control
R^2	coefficient of determination
RE	relative error
RSD	relative standard deviation
SD	standard deviation
SIM	selected ion monitoring
TFAA	trifluoroacetic acid anhydride
WB	whole blood

Table 1 Influence of the hematocrit on the GHB concentration measured in DBS samples, using GC-MS, operating in SIM mode. Values indicate the % deviation from the GHB 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.

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

Table 2 Calibration and sensitivity data for the determination of GHB in DBS using GC-MS in SIM mode.

Slope n=4; mean ± SD (95 % CI)	Intercept n=4; 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

Table 3 Inter- (n=4; in duplicate) and intra- (n=6) batch precision and accuracy data for the quality control of GHB determination in DBS samples prepared in whole blood. Intra-batch data were obtained from blood with intermediate hematocrit.

		GHB concentration measured (µg/ml)			Precision (% RSD)			Accuracy (% nominal concentration)		
	Hematocrit ------------------ Nominal GHB Concentration (µg/ml)	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		

[*] For one day of calibration, single analysis was performed of these QC's because of sample loss during sample preparation

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

Hematocrit	Back-calculated GHB concentration (n=3; in duplicate, μg/ml)	Inter-batch precision (% relative standard deviation, n=3; in duplicate)	Accuracy (% nominal concentration, n=3; in duplicate)
Low (0.38)	191	6.57	95.6
Intermediate (0.45)	210	6.10	105
High (0.50)	207	5.54	103

Table 5 GHB level (µg/ml) in paired venous and capillary DBS, and venous WB samples in 7 patients presenting at the emergency department with a suspected GHB-intoxication. A DBS (single analysis) was analyzed using "on spot" derivatization and GC-MS, operating in SIM mode, while venous WB (in duplicate) was analyzed according to the procedure of Van hee *et al.* [20].

GHB-positive case N°	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

* We suspect this higher value to be due to contamination of the fingertip.

Fig. 1 Average % bias *vs.* blood volume spotted for the determination of GHB in DBS. The DBS (n=5 or 6, at low and high nominal value) were analyzed using "on spot" derivatization and GC-MS, operating in SIM mode. Dotted lines indicate the ± 15 % (bias) limits.

Fig. 2 Representative chromatogram obtained after analysis of a 6-mm disc punched out from a DBS prepared from blood spiked with GHB at 2 µg/ml (LLOQ). Five µl of a 25 µg/ml IS solution was added to the punch before derivatization and analysis with GC-MS operating in SIM mode.

Fig. 3 Long-term stability of GHB in DBS (n=6, low and high GHB concentration level) stored at room temperature in a zip-closure plastic bag with desiccant up to 148 days. The average % bias *vs.* time point of DBS analysis (days) is plotted and needed to be within the ± 15 % limits, indicated by the dotted lines. T_0 refers to time point zero (DBS analysis after 2 hours of drying).

Fig. 4 Overlay of representative chromatograms obtained by analyzing the derivatized extract of the capillary and venous DBS, collected from the first patient who tested positive for GHB use (GHB-positive case N° 1 in Table 5), by using "on spot" derivatization and GC-MS, operating in SIM mode.

Fig. 1

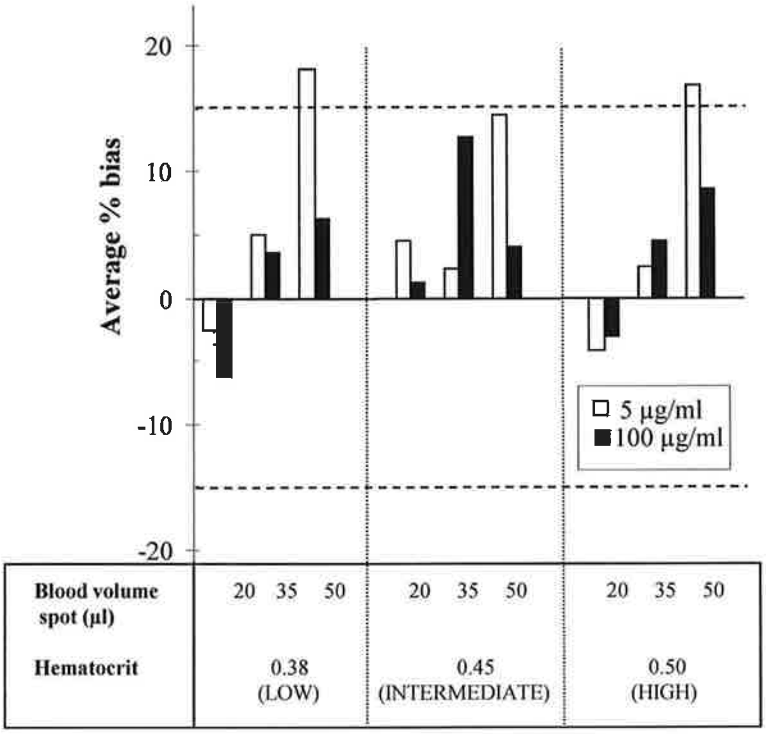

Average % bias
20
10
0
-10
-20
□ 5 µg/ml
■ 100 µg/ml
Blood volume spot (µl)
20 35 50
20 35 50
20 35 50
Hematocrit
0.38 (LOW)
0.45 (INTERMEDIATE)
0.50 (HIGH)

Fig. 2

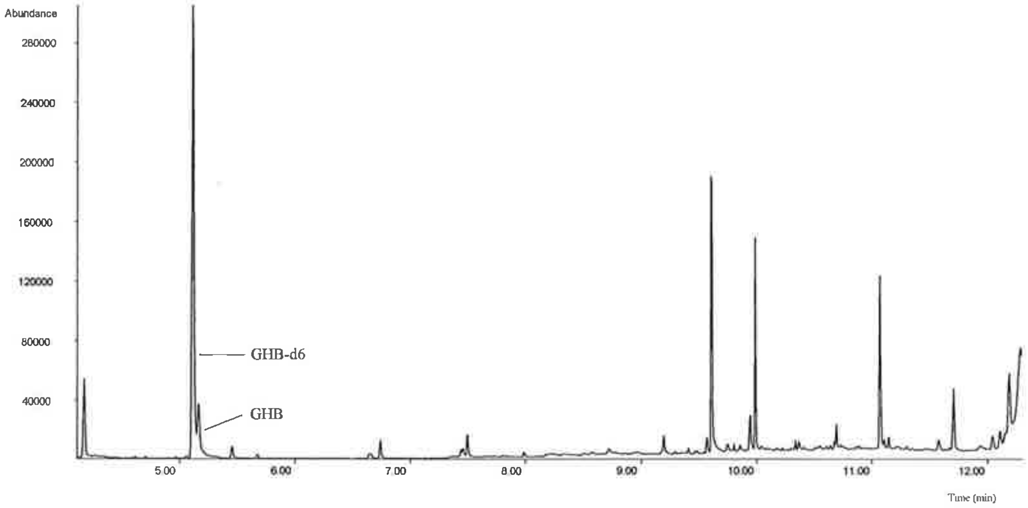

Abundance
280000
240000
200000
160000
120000
80000
40000
GHB-d6
GHB
5.00
6.00
7.00
8.00
9.00
10.00
11.00
12.00
Time (min)

Fig. 3

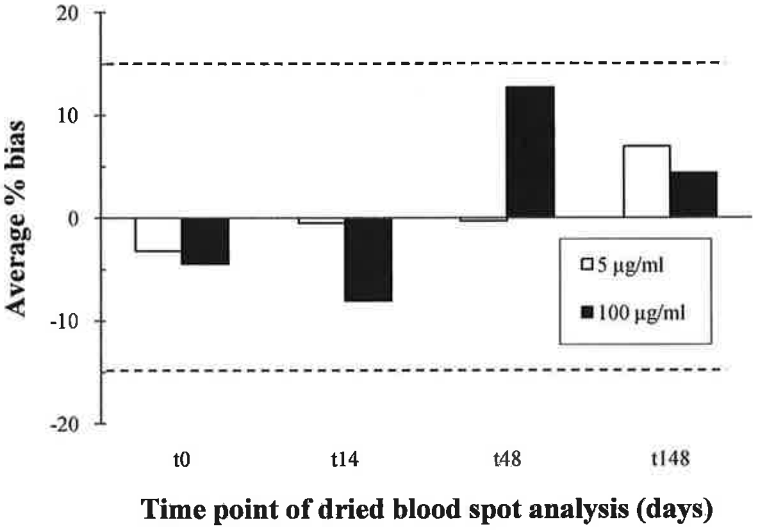
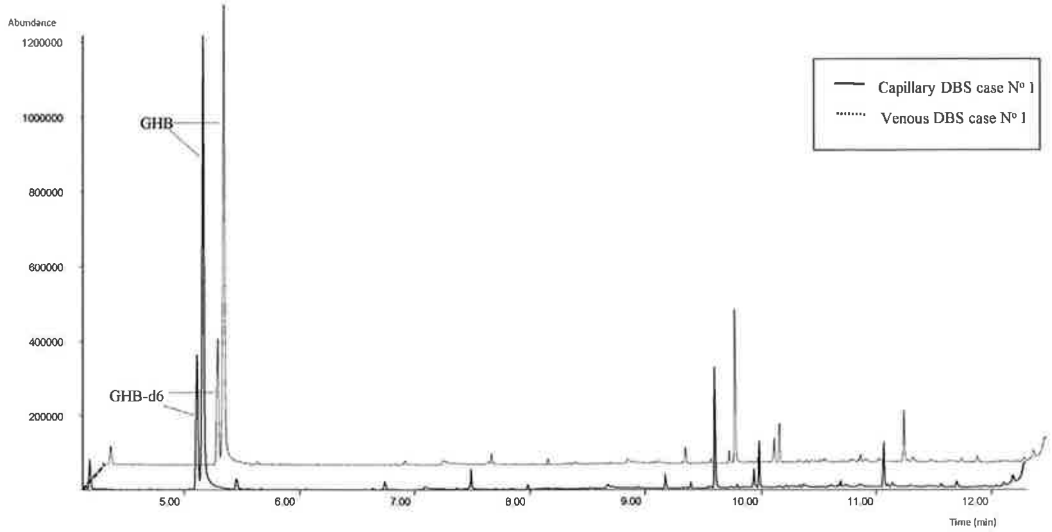

20
10
0
-10
-20
Average % bias
□5 µg/ml
■100 µg/ml
t0
t14
t48
t148
Time point of dried blood spot analysis (days)

Fig. 4


Abundance
1200000
1000000
800000
600000
400000
200000
GHB
GHB-d6
Capillary DBS case Nº 1
Venous DBS case Nº 1
5.00
6.00
7.00
8.00
9.00
10.00
11.00
12.00
Time (min)

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

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