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Do capillary dried blood spot concentrations of gamma-hydroxybutyric acid mirror those in venous blood? A comparative study

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Abstract

Gamma-hydroxybutyric acid (GHB) is a well-known illicit club and date-rape drug. Dried blood spot (DBS) sampling is a promising alternative for classical venous sampling in cases of (suspected) GHB intoxication since it allows rapid sampling, which is of interest for the extensively metabolized GHB. However, there is limited data if -and how- capillary DBS concentrations correlate with venous concentrations. We conducted a comparative study in 50 patients with suspected GHB intoxication, to determine and to correlate GHB concentrations in venous DBS (vDBS) and capillary DBS (cDBS). This is the first study that evaluates in a large cohort the correlation between capillary and venous concentrations of an illicit drug in real-life samples. Of the 50 paired samples, 7 were excluded: the vDBS concentration was below the LLOQ of 2 μg/mL in 3 cases and 4 samples were excluded after visual inspection of the DBS. Bland-Altman analysis revealed a mean % difference of -2.8% between cDBS and vDBS concentrations, with the zero value included in the 95% confidence interval of the mean difference in GHB concentration. A paired sample t-test confirmed this observation ($p=0.17$). Also the requirement for incurred sample reproducibility was fulfilled: for more than 2/3 of the samples the concentrations obtained in cDBS and those in vDBS were within 20% of their mean. Since equivalent concentrations were observed in cDBS and vDBS, blood obtained by fingerprick can be considered a valid alternative for venous blood for GHB determination.

Introduction

Gamma-hydroxybutyrate (GHB) and its precursor gamma-butyrolactone (GBL) are used as recreational drug and date-rape drug, particulary in a nightclub environment and in men having sex with men. There have been many cases of GHB intoxication, also in combination with other drugs of abuse.[1-4] Toxicological interpretation of GHB concentrations in clinical and forensic samples is impeded by its endogenous presence. Furthermore, given the short half-life of GHB, rapid sampling is needed.[5] Dried blood spot (DBS) sampling has been proposed as a possible alternative for classical venepuncture, offering several advantages, in particular rapid and easy collection of a representative sample and easy sample transfer and storage.[6] A number of studies have provided a proof-of-concept of the potential to determine abused substances, including GHB, in DBS samples.[7] However, these studies have generally not assessed the developed methodology in a large cohort of patients, comparing capillary samples with the gold standard of venous samples. In addition, in most reports a cross-comparison between venous DBS (vDBS) and capillary DBS (cDBS) concentrations is lacking.[7] Therefore, although DBS sampling is an interesting and promising alternative to classical venous sampling, it remains to be demonstrated if cDBS concentrations correlate with those in venous samples. While we previously demonstrated that GHB can be quantitatively determined in DBS of patients with a GHB/GBL intoxication and in narcoleptic patients taking the medication Xyrem® (sodium oxybate, the sodium salt of GHB)[8-10], the aim of this study was to compare GHB concentrations in vDBS and cDBS in a large cohort of patients with acute GHB intoxication.

Materials and methods

With the exception of the sodium fluoride/potassium oxalate (NaF-KOx) blood collection tubes (9 mL tubes with 100 mg sodium fluoride and 22.50 mg potassium oxalate obtained from Terumo UK Ltd), all materials were provided from previously published suppliers.[8]

Samples

This study was approved by The UK National Research Ethics Service (Reference 11/LO/0976). All patients presenting to the Emergency Department at St Thomas' Hospital, London with either a clinical diagnosis or other indications of GHB/GBL intoxication were considered for inclusion. cDBS were generated by non-volumetric direct application of a blood drop from the fingertip onto Whatman filter paper following a fingerprick. vDBS were obtained by pipetting 25 µL of venous blood from the NaF-KOx blood tubes in which the venous samples were collected and stored. DBS were dried for at least 2 hours before storage at room temperature in zip-closure plastic bags with desiccant. Paired cDBS and venous whole blood samples were collected at the time of admission from 99 patients. As these patients were generally drowsy or agitated at presentation, informed consent was not possible prior to the collection of samples and therefore delayed consent was employed. Of the patients sampled, 50 provided delayed consent. The paired samples obtained from these 50 patients were used in this study.

Analytical procedures

GHB concentrations in DBS were determined as described before, using a validated GC-MS-based procedure.[9] Briefly, 6-mm partial-spot punches were taken from the DBS. After adding the internal standard GHB-d6, the DBS punches were subjected to "on-spot derivatization" by direct application of a mixture of 50 µL trifluoroacetic acid anhydride and heptafluorobutanol (2:1) and heating for 10 min at 60°C. After evaporation under a gentle stream of nitrogen, the dried extract was re-dissolved in 100 µL ethylacetate and 1 µL was injected into an Agilent 6890 GC system coupled to a 5973 mass spectrometer. Of those DBS with a GHB concentration above 100 µg/mL, 10 µL of the final derivatized extract was diluted to 100 µL with ethyl acetate. The capillary column was a 30 m x 0.25 mm i.d. x 0.25 µm Agilent HP-5MS column. The injection temperature was set at 250°C, and a splitless injection was performed with a purge time of 1.5 min. The initial column temperature was set at 60°C for 1.5 min, ramped at 10°C/min to 110°C, then raised by 50°C/min to 300°C, which was held for 2 min, resulting in a total analysis time of 12.3 min. High-purity helium was used as the carrier gas with a constant flow rate of 1.3 mL/min. The transfer line temperature and ion source temperature were set at 280 and 230°C, respectively. MS quadrupole temperature was set at 150°C and ionization energy of 70 eV was used. Quantification of GHB and GHB-d6 was performed in the selected ion monitoring (SIM) mode using *m/z* 155, 183, 227 and 242 for derivatized GHB and 161, 189, 231 and 245 for derivatized GHB-d6.

Storage experiment

To evaluate GHB stability in venous whole blood (collected in NaF-KOx tubes), we added two different concentrations (10 and 100 µg/mL) in whole blood and stored the samples at different temperatures (4°C and room temperature) for up to 2 weeks. At different time points, 25-µL DBS were generated from this spiked blood, which were analyzed using the above-mentioned DBS-based GC-MS method.

Data analysis

To evaluate the correlation between venous and capillary concentrations, we performed a paired sample t-test, using Excel, and Bland-Altman and Passing-Bablok analysis, using MedCalc® (MedCalc software bvba, Ostend, Belgium).

A p-value ≤ 0.05 was considered statistically significant. In the Bland-Altman plot, the differences between venous and capillary concentrations were plotted against the average of both measurements. Indicated in this plot are the mean difference between both concentrations and the limits of agreement (1.96 SD), along with the respective 95% confidence intervals. In the Passing-Bablok scatter plot diagram, capillary concentrations were plotted against venous concentrations. If the confidence interval of the slope includes 1, there is no proportional difference between both measurements. When the confidence interval of the intercept value includes 0, it can be concluded that there is no systematic difference between both methods.

Results and discussion

Of the 50 paired samples, 7 were excluded: the vDBS concentration of 3 samples was below the LLOQ of 2 µg/mL, while 4 samples were excluded after visual inspection of the DBS (in 3 cases cDBS samples were too small (< 6 mm diameter) and in one case vDBS had an irregular shape). cDBS concentrations of GHB ranged from 41 to 646 µg/mL, whereas vDBS concentrations ranged from 48 to 705 µg/mL. Clinically, all patients - apart from the 3 patients with a vDBS concentration below LLOQ - had clinical symptoms consistent with acute GHB intoxication.

In literature, a cut-off GHB concentration of 4-5 µg/mL in blood has been proposed to differentiate between endogenous and exogenous GHB.[5,11-13] In our study, 43 patients were screened positive for GHB: both venous and capillary concentrations were well above these cut-off levels. In the 3 cases of our study that did not have symptoms of acute GHB intoxication, vDBS concentrations were below the limit. However, GHB intake is likely as clearly higher signals were detected than those found in GHB-naïve persons (see Figure 1). These cases presented to the hospital with clinical symptoms consistent with recent stimulant use or secondary to GHB withdrawal rather than acute intoxication. In all 3 cases, there was some evidence that GHB use may have taken place more than 5 hours before sampling (e.g. history of GHB abuse, declared GHB use,…). However, it was not possible to formally confirm GHB use in these patients, as urine samples were not routinely collected. As suggested by these 3 cases, rapid sampling is of utmost importance since GHB is metabolized rapidly, with a half-life of less than one hour, resulting in blood concentrations below proposed cut-off levels within a few hours after use.[14,15] Shima and colleagues proposed lowering the cut-off to 1 µg/mL in blood in cases where in-life blood specimens can be collected aseptically and stored at 4°C or lower before timely analysis.[16] In legal cases, cDBS samples may provide the advantage that they may not only allow rapid sampling, but also stabilise the sample, allowing long-term storage at room temperature.[9]

We performed Bland-Altman analysis to compare cDBS and vDBS concentrations. As can be seen in Figure 2a, we found a mean % difference of -2.8%, with the zero value included in the 95% confidence interval of the mean difference in GHB concentration. From this, it can be concluded that there is no significant difference between cDBS and vDBS concentrations. A paired sample t-test confirmed this conclusion (p=0.17). We also applied the European Medicine Agency (EMA) guideline for incurred sample reanalysis in bioanalytical method validation.[17] These state that the concentration obtained for the initial analysis and the concentration obtained by reanalysis should be within 20% of their mean for at least 67% of the repeats. Although this requirement actually concerns reanalysis of the *same* samples, this condition was still fulfilled when analyzing *different* (cDBS *versus* vDBS) samples: in 72% of cases the concentrations obtained in cDBS and those in vDBS were within 20% of the mean GHB concentrations obtained with the 2 methods. As can be seen in Figure 2b, a Passing-Bablok scatter plot also demonstrated a good overall correlation between cDBS and vDBS

GHB concentrations, although 1 was just not included in the 95% confidence interval of the slope.

In addition to performing a pairwise comparison between cDBS and vDBS, we also performed reanalysis of both cDBS (n=29) and vDBS (n=28). Again, the EMA requirement for incurred sample reanalysis was fulfilled: we found that for more than two-thirds (i.e. 70%) of the samples the initial concentration and the concentration obtained by reanalysis, were within 20% of the mean of the first and repeat measurement.

It should be noted that, while cDBS and venous blood were sampled at almost the same time point, in some cases, there was a delay of several days before vDBS were prepared from the venous blood. To exclude that this may have an effect on our results, we evaluated GHB stability in spiked venous whole blood collected in NaF-KOx tubes that were stored for up to two weeks at 4°C and at room temperature before DBS preparation. The bias from nominal concentrations (10 or 100 μg/mL) did not exceed 11%. This suggests that no substantial alteration takes place in GHB concentration if venous blood, collected in NaF-Kox tubes, is stored under these conditions. Although this finding is consistent with previously published data[18-20], it should be noted that in incurred (real-life) samples a contribution from e.g. hydrolysis of GHB glucuronide might not be excluded. However, such contribution is expected to be limited.[21]

Conclusion

DBS sampling is a promising alternative for classical venous sampling in cases of (suspected) GHB intoxication: the DBS sampling technique allows rapid sampling -which is of interest for the extensively metabolized GHB- and DBS are also easier to store and transport than venous samples. The study reported here is the largest comparative study to date evaluating capillary and venous concentrations of an illicit drug. In a large cohort of patients with acute GHB intoxication we observed equivalent GHB concentrations and an excellent correlation between cDBS and vDBS. In conclusion, blood obtained by fingerprick is a valid alternative for venous blood for GHB determination.

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Figure legends

Figure 1: Overlay of representative chromatograms obtained by analyzing (a) vDBS of a GHB-naïve person, (b) vDBS of a patient with assumed GHB intake (GHB concentration < 2 µg/mL), (c) vDBS with a GHB concentration of 2 µg/mL (LLOQ), (d) vDBS of a GHB-intoxicated person (GHB concentration of 48 µg/mL), using a DBS-based GC-MS method[9].

Figure 2: (a) Bland-Altman analysis of cDBS and vDBS, plotting the % differences between both GHB concentrations (y-axis) against the average of these results (x-axis). The mean difference and the limits of agreement (set to 1.96 SD) are also indicated with its 95% confidence interval. (b) Passing-Bablok regression analysis of cDBS and vDBS, plotting the concentrations in both matrices against each other. The solid line illustrates the regression line, the dashed lines indicate the confidence interval for the regression line and the dotted line corresponds to the identity line.