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ORIGINAL ARTICLE

Determination of ethyl glucuronide and ethyl sulfate from dried blood spots

Ana Hernández Redondo · Alexandra Schroeck · Beat Kneubuehl · Wolfgang Weinmann

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Abstract
Background Ethyl glucuronide (EtG) and ethyl sulfate (EtS) are non-oxidative minor metabolites of ethanol. They are detectable in various body fluids shortly after initial consumption of ethanol and have a longer detection time frame than the parent compound. They are regarded highly sensitive and specific markers of recent alcohol uptake. This study evaluates the determination of EtG and EtS from dried blood spots (DBS), a simple and cost-effective sampling method that would shorten the time gap between offense and blood sampling and lead to a better reflectance of the actual impairment.
Methods For method validation, EtG and EtS standard and quality control samples were prepared in fresh human heparinized blood and spotted on DBS cards, then extracted and measured by an LC-ESI-MS/MS method. Additionally, 76 heparinized blood samples from traffic offense cases were analyzed for EtG and EtS as whole blood and as DBS specimens. The results from these measurements were then compared by calculating the respective mean values, by a matched-paired *t* test, by a Wilcoxon test, and by Bland–Altman and Mountain plots.
Results and discussion Calibrations for EtG and EtS in DBS were linear over the studied calibration range. The precision and accuracy of the method met the requirements of the validation guidelines that were employed in the study. The stability of the biomarkers stored as DBS was demonstrated under different storage conditions. The *t* test showed no significant difference between whole blood and DBS in the determination of EtG and EtS. In addition, the Bland–Altman analysis and Mountain plot confirmed that the concentration differences that were measured in DBS specimens were not relevant.

Keywords Ethyl glucuronide · Ethyl sulfate · Alcohol markers · Dried blood spots · LC-ESI-MS/MS · Method comparison

A. Hernández Redondo · A. Schroeck · B. Kneubuehl · W. Weinmann
Institute of Forensic Medicine, University of Bern, Bern, Switzerland

A. Hernández Redondo · A. Schroeck · B. Kneubuehl · W. Weinmann (✉)
Institut für Rechtsmedizin, Medizinische Fakultät, Universität Bern, Bühlstrasse 20, 3012 Bern, Switzerland
e-mail: wolfgang.weinmann@irm.unibe.ch

Introduction

Ethyl glucuronide and ethyl sulfate are direct alcohol markers which are widely used for clinical, forensic and traffic cases, and in workplace monitoring [1–3]. Apart from being highly sensitive and specific in comparison with other traditional biomarkers [4, 5], they are detectable in various matrices, each of them with a different detection window. Depending on the ethanol uptake, ethyl glucuronide (EtG) and ethyl sulfate (EtS) are detectable in blood for up to 8 h, in urine for up to 4 days [6–9], and in hair for up to several months depending on the hair length [10], even when ethanol is no longer detectable. For those reasons, EtG and EtS are getting more and more popular not only for the detection of recent alcohol consumption (blood, urine) [8] but also for the determination of chronic alcohol abuse (hair, nails) [11, 12].

For decades, filter paper was used to collect and analyze human blood in form of dried blood spots (DBS) for neonatal metabolic screening [13, 14]. However, it was only in the last two decades that the method was accepted for therapeutic drug monitoring [15, 16] and the detection of drugs of abuse [17–19]. The advantages of DBS include the small volume of sample that is necessary (between 10 and 100 μL), the less invasive sample collection method, and an easier and more economic transport and storage of specimens: cooling or

special biohazard arrangements are not necessary due to the stability of most analytes in DBS and the reduction of a virus infection risk [20–22]. Despite the advantages of DBS over conventional whole blood analysis, some doubts have arisen in the scientific community about the viability of the method for quantitative determination of drug concentration in a regulated environment [23]. The most delicate factors of the method are the influence of the hematocrit value and the application mode of the isotopically labeled internal standards on the determination of the analytes [24–26]. Some authors also distrust some of the so-called advantages of the DBS method since the small sample volumes require very sensitive instruments for determination of the analytes and also see challenges with incurred sample reanalysis.

In this study, the viability of the DBS method for EtG and EtS determination was evaluated following some recent reports about the applicability of this technique for monitoring alcohol consumption [27, 28] and in analogy to a recent publication where EtG and EtS were determined from dried urine spots [29]. Further, the method was validated according to the guidelines of the German Society of Toxicological and Forensic Chemistry (GTFCh) [30]. A statistical comparison was also performed with 76 heparinized blood samples from traffic offense cases that were analyzed as whole blood and as DBS.

Experimental

Analytical methods

Preparation of calibration and quality control samples

Stock solutions of EtG and EtS (1 mg/mL) were prepared in pure methanol and used to prepare the working solutions also in methanol for spiking of calibration and quality control (QC) samples. Calibration standards were prepared on a daily basis by spiking blank human whole blood at concentration levels of 0.1, 0.2, 1.0, 2.5, 5.0, and 10 μg/mL. Quality control samples were prepared at EtG and EtS concentrations of 0.3 μg/mL (QC_{low}) and 6.6 μg/mL (QC_{high}).

Processing of whole blood samples

Fifty microliters of blood (calibrators and samples) was transferred to an Eppendorf tube and 150 μL of acetonitrile that contained internal standards (IS) (0.66 μg/mL pentadeuterated EtG (D_5-EtG), ca. 0.05 μg/mL D_5-EtS) was added. The samples were mixed by vortexing for 10 min and finally centrifuged for 10 min at 18,320×*g*. One hundred microliters of the supernatant solutions were then transferred into 200-μL restricted volume autosampler glass vials and evaporated under a gentle stream of nitrogen at 50 °C. The residues were reconstituted with 100 μL of a water/acetonitrile/formic acid (95/5/0.1; *v*/*v*) solution and shaken for 1 min before injection of 10 μL into the LC-MS/MS system.

Preparation and processing of DBS samples

For each sample (calibration, quality control, unknown), 10 μL of blood (instead of 50 μL in whole blood samples) was pipetted onto the center of a printed circle on 226-1004 Bioanalysis cards, where they diffused and dried for at least 3 h at ambient room temperature prior to further analysis. The whole DBS was punched out using a 1-cm-diameter hole puncher and collected in an Eppendorf tube. The extraction of the analytes was performed by adding 500 μL of methanol containing IS (0.03 μg/mL D_5-EtG, ca. 0.005 μg/mL D_5-EtS) and subsequent mixing by vortexing for 5 min. The methanolic solution was transferred to a vial and dried under a gentle stream of nitrogen at 50 °C. The samples were then reconstituted with 50 μL of a water/acetonitrile/formic acid (95/5/0.1; *v*/*v*) solution and shaken for 1 min. The reconstituted solution was transferred into restricted volume vials and 10 μL was injected into the LC-MS/MS system.

Chromatographic system

The chromatographic system and conditions employed for the analysis were described earlier [29]. Instrumentation consisted of a CTC PAL autosampler, an Agilent 1200 series HPLC, and a QTrap 3200 mass spectrometer (AB Sciex, Rotkreuz, Switzerland) controlled by Analyst 1.5.1 software, which was also used for the data acquisition and processing. For the analytical separation, a 150×2-mm column with 4 μm particles (Synergi Polar-RP, Phenomenex, Torrance CA, USA) contained in a column heater at 40 °C was used. To enhance signal intensity, 2-propanol was added post-column with a flow of 0.3 mL/min by a T-union before the eluent enters the electrospray ion source.

The gradient elution was performed with 0.1 % formic acid in water as mobile phase A and 0.1 % formic acid in acetonitrile as mobile phase B. The gradient was run at a flow of 0.3 mL/min starting at 2 % B for 2 min and increasing to 90 % B for 3 min. The gradient was then changed back to the starting conditions over 0.5 min and kept constant for 6.5 min to re-equilibrate. The retention times of EtG and EtS were 1.96 and 1.66 min, respectively.

The mass spectrometer was operated in the negative multiple reaction monitoring mode by employing a TurboIonSpray source. The following transitions were monitored: EtG: *m*/*z* 221/75 as quantifier, *m*/*z* 221/85 as qualifier, and *m*/*z* 226/75 for the deuterated internal standard. EtS: *m*/*z* 125/97 as quantifier, *m*/*z* 125/80 as qualifier, and *m*/*z* 130/98 for the deuterated internal standard.

Chemicals and materials

EtG and D_5-EtG were obtained from Lipomed (Arlesheim, Switzerland). Sodium EtS was purchased from ABCR (Karlsruhe, Germany). Deuterated EtS (D_5-EtS) was synthesized by an in-house procedure with a purity of >99.9 % [31]. Acetonitrile supra gradient grade was obtained from Biosolve (Chemie Brunschwig, Basel, Switzerland) and HPLC-grade water was produced in-house with a Milli-Q water system from Millipore (Billerica, USA). Formic acid (puriss p.a., 98 %), methanol (spectrophotometric grade, ≥99 %), and MiniPax Sorbent packages were purchased from Sigma-Aldrich (Buchs, Switzerland). Bioanalysis cards number 226-1004 from PerkinElmer (Greenville, USA) were used for dried blood spot collection and a home-made 1-cm-diameter hole puncher was employed to cut out the dried blood spots. Nine milliliters heparin S-Monovette was purchased from Sarstedt (Nümbrecht, Germany). Drug-free lithium heparin whole blood was obtained from six volunteers who were abstinent from alcohol for at least 2 weeks.

Method validation

The analytical method for the determination of EtG and EtS in DBS was validated according to the guidelines of the GTFCh [30]. During the validation, all samples were processed as DBS as described before. The evaluated parameters were linearity, accuracy and precision, selectivity, stability, and extraction efficiency. The accuracy was determined by calculating the percent deviation of the data from the nominal concentration. The precision, which is usually expressed as "imprecision," was determined by calculating the relative standard deviation (% RSD) [30].

The linearity of the method was tested by analyzing a six-point calibration curve constructed on six different days in the range 0.1–10 μg/mL. The intra- and inter-day precision and accuracy were studied by analyzing replicate samples (n=2) at two different concentration levels (0.3 and 6.6 μg/mL) over eight different days (n=8). The selectivity of the method was studied by analyzing blank blood from six different sources.

The stability of EtG and EtS in DBS was investigated by storing collection cards with DBS spiked at 0.3 μg/mL and at 6.6 μg/mL in sealable plastic bags that contained desiccant packages at room temperature and at 4 °C for 1, 2, and 3 weeks, respectively. The desiccant packages in the plastic bags were replaced with new ones every week to protect the specimens against humidity and moisture. For the two investigated QC levels and storage conditions, six replicates (n=6) were analyzed on the first day and after the different time periods given before.

The extraction efficiency of the method was calculated by comparing the peak area ratios of the analytes/internal standards (percent) between extraction and control samples at each QC level. The control samples (n=6) were prepared by extracting 10 μL of blank DBS samples with 500 μL of methanol. Two hundred microliters of the methanolic solution was then transferred to a vial and 500 μL of the MeOH/IS solution and 10 μL of a quality control sample prepared in methanol were added. The mixtures were then evaporated under a gentle stream of nitrogen at 50 °C and reconstituted with 50 μL of a water/acetonitrile/formic acid (95/5/0.1; v/v) solution. The extraction samples (n=6) were prepared by extracting 10 μL of spiked DBS samples with 500 μL of methanol. Two hundred microliters of the methanolic solution was then transferred to a vial and 500 μL of the MeOH/IS solution was added. The mixtures were then evaporated under a gentle stream of nitrogen at 50 °C and reconstituted with 50 μL of a water/acetonitrile/formic acid (95/5/0.1; v/v) solution.

Method comparison

To evaluate the DBS method for determination of EtG and EtS, 76 heparinized blood samples from traffic offense cases with blood alcohol concentrations ranging from 0.48 to 3.03 ‰ were analyzed as whole blood and as DBS samples with a separate calibration for each analysis method. The mean and median values of the results from each method were compared for EtG and EtS. Further, the normal distribution of the differences was tested using the Kolmogorov–Smirnov test, and a matched-paired t test and Bland–Altman analysis [32, 33] (which rely on the assumption of normally distributed data) were performed. In addition, two further statistical tests, which are valid for data from any distribution, were performed: Wilcoxon test and Mountain plot [34]. The matched-paired t test and Wilcoxon test show if there is a significant mean difference between two sets of paired data, whereas the Bland–Altman analysis and Mountain plot offer simple and graphical methods to estimate the analytical error [34].

Results

Validation of the DBS method

The calibration curves were linear in the measured range 0.1–10 μg/mL and showed a mean correlation coefficient of 0.9980 and 0.9984 for EtG and EtS, respectively. They were weighed ($1/x$) in order to avoid the data at the high end of the calibration curve to dominate the calculation resulting in excessive error in the low calibration range. The intra- and inter-day precision and the accuracy met the requirements of the GTFCh for validation of analysis methods for both QC levels (Table 1). The lowest calibration level, 0.1 μg/mL, was measured with an accuracy and precision (% RSD)

Table 1 Precision (relative standard deviation, % RSD) and accuracy (percent deviation from the nominal concentration) of the DBS method determined at low (0.3 μg/mL) and high (6.6 μg/mL) EtG and EtS concentrations

Nominal concentration	EtG		EtS	
	Low	High	Low	High
Intra-day precision (% RSD, n=2)	6.1	4.4	6.6	6.5
Inter-day precision (% RSD, n=8)	7.5	7.6	6.3	7.8
Accuracy (n=16)	107.3	106.9	108.8	106.9

smaller than 20 % and was therefore selected as the lowest limit of quantification of the method (results not shown).

The selectivity of the method was studied by analyzing blank blood from six different sources. No interfering peaks for EtG or EtS were observed in any of the studied blank DBS samples.

The stability of EtG and EtS in DBS was investigated after storing the cards at room temperature and at 4 °C for 1, 2, and 3 weeks, respectively, as described in the experimental part. The results shown in Table 2 demonstrate that EtG and EtS in DBS are stable for at least 3 weeks when stored either at room temperature or at 4 °C: the mean percentages found were within 85–115 % of the results obtained when the samples were analyzed after a 3-h drying period.

The extraction efficiency was assessed by comparing the peak area ratios of extracted and control QC samples at low and high level (n=6) as described before. The extraction efficiency for EtG and EtS from DBS was 43 and 48 %, respectively, for both tested concentration levels. The reason for this relative low extraction efficiency could be the irreversible binding of the analytes to the filter paper since, as observed in Table 2, no decomposition is observed after 3 weeks of storage neither at room temperature nor at 4 °C.

Table 2 Stability data of EtG and EtS in DBS stored at room temperature and at 4 °C for 1, 2, and 3 weeks, respectively, presented as the mean percentage±standard deviation of the results obtained when analyzed on the first day

	RT		4 °C	
	Low	High	Low	High
EtG				
1 week	102.3±8.1	93.0±6.6	101.5±6.1	98.6±9.0[a]
2 weeks	100.0±8.7	105.0±6.5	104.3±6.3	108.4±2.8
3 weeks	111.4±4.8[b]	105.0±5.5	115.2±8.4[b]	109.6±7.1
EtS				
1 week	102.6±5.5	93.6±5.7	102.4±7.0	93.8±6.8
2 weeks	105.6±4.9	103.6±7.1	111.8±3.3	105.3±2.2
3 weeks	108.8±5.1[b]	103.1±3.6	108.7±6.1[b]	102.6±7.1

[a] n=5

[b] n=4

Comparison of the results from whole blood and from DBS samples

The EtG and EtS concentrations in blood ranged from 460 to 6,250 ng/mL (mean 2,179 ng/mL, median 1,885 ng/mL) and from 200 to 2,720 ng/mL (mean 1,157 ng/mL, median 1,020 ng/mL), respectively. In DBS, the EtG and EtS concentrations ranged from 428 to 6,690 ng/mL (mean 2,126 ng/mL, median 1,885 ng/mL) and from 161 to 2,680 ng/mL (mean 1,177 ng/mL, median 1,085 ng/mL), respectively.

The normal distribution of the differences was tested and proofed for both analytes using the Kolmogorov–Smirnov test (results not shown). Then, a matched-paired t test was conducted in order to assess if there was a significant difference between the two methods in the determination of EtG and EtS. The paired t test value calculated for EtG and EtS was 1.982 and 1.718, respectively, lower than the t score for a two-tailed distribution for a significance level of 0.05, 1.991. Thus, according to this test, there was no significant difference between the two methods for any of the studied analytes.

In addition, the Wilcoxon rank sum test was performed which is valid for data from any distribution, whether normal or not, and for this reason less meaningful than the t test. The calculated test and threshold values for a significance level of 0.05 were 376 and 371, respectively, for EtG, and 456 and 364, respectively, for EtS. For both biomarkers, the test values calculated with this test were higher than the threshold values, indicating a significant difference in the distribution of the differences.

Apart from these statistical tests, the Bland–Altman analysis was used to assess the agreement between the two methods for EtG and EtS measurement and to determine if the two methods match enough to be interchanged [32]. Taking into consideration the normal distribution of the differences, as proofed by the Kolmogorov–Smirnov test, the mean difference of the methods, the standard deviation (SD), the limits of agreement, and the confidence intervals (CI) were calculated as described in [33]. The limits of agreement show a range of values where 95 % of the differences are expected to lie, whereas the confidence intervals show a range in which the population values lie with 95 % probability [33]. In order to estimate the impact of the measured differences on the EtG and EtS analysis, these

parameters were calculated relative to the mean of the two values, so the results are shown in percent.

The Bland–Altman plot in Fig. 1 shows a graphical representation of the between-method percentage difference (*y*-axis) versus the average of the two methods (*x*-axis), since this is the best estimate of the true value [32]. For EtG, the mean difference between the methods was 2.92 % (SD 9.48 %), with limits of agreement of −15.7 and 21.5 %. For EtS, the mean difference between the methods was −1.79 % (SD 8.44 %), with limits of agreement of −18.3 and 14.8 %. The mean percentage difference and the 95 % limits of agreement are graphically represented in Fig. 1 as solid and dotted lines, respectively. The CI with a 95 % probability for the mean percentage differences and for the limits of agreement of EtG and EtS are shown in Table 3, but not graphically represented for clarity reasons.

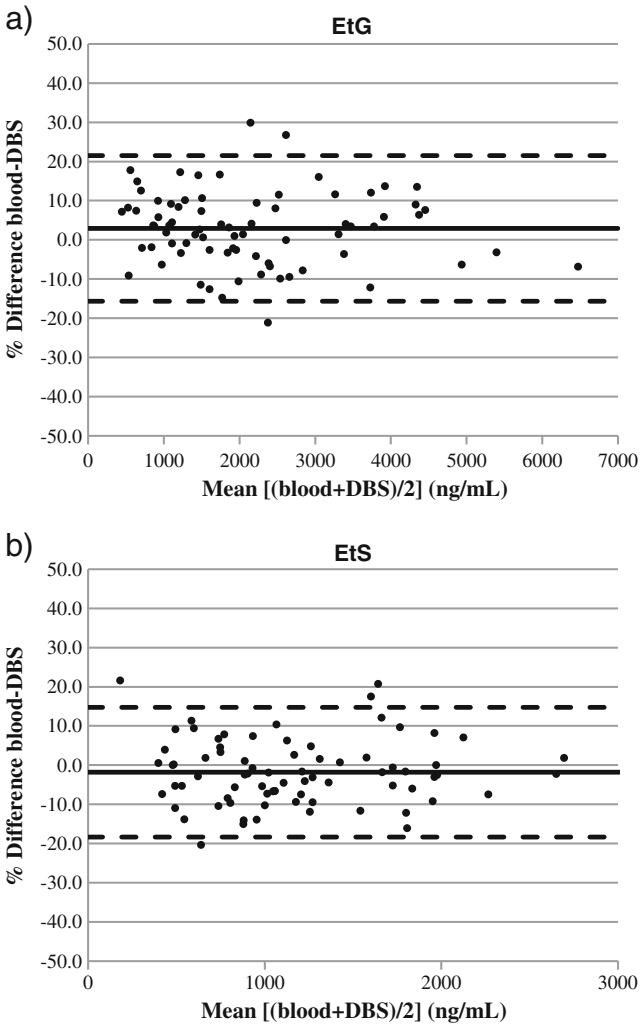

Fig. 1 Bland–Altman difference plots for EtG (**a**) and EtS (**b**) of the percentage differences between whole blood and DBS assays (n=76) against the average obtained by the two assays. The *solid lines* are the mean percentage differences, whereas the *dashed lines* indicate the limits of agreement set to ±1.96 SD

Table 3 Confidence intervals (CI) for the mean percentage differences and limits of agreement of EtG and EtS

	EtG (%)	EtS (%)
$CI_{mean\ difference}$	0.76 to 5.09	−3.72 to 0.14
$CI_{+1.96\ SD}$	17.8 to 25.3	11.4 to 18.1
$CI_{-1.96\ SD}$	−19.4 to −11.9	−21.7 to −15.0

In addition to the Bland–Altman analysis, and as recommended in the literature [34], the Mountain plot of the percentage differences of EtG and EtS, also called "folded empirical cumulative distribution," was performed and is shown in Fig. 2. For EtG, the Mountain is centered at 3.4 %, and 95 % of the differences lie between −15 and 27 %. For EtS, the Mountain is centered at −2.5 %, with 95 % of the differences between −16 and 20 %.

Discussion

In the study presented here, the influence of the hematocrit effect on the accurate quantification of the analytes was overcome by collecting a fixed volume of blood on the filter cards and then punching a hole larger than the blood spots [15, 35]. Only 10 μL of blood was used for the analysis of these two alcohol biomarkers. This is a big reduction of the amount of required blood compared to the whole blood method where 50 μL of blood or more is necessary. To compensate this sample down scaling, a concentration step was introduced when reconstituting the extracts in order to be able to quantify low EtG and EtS concentrations. In addition, during method development, the optimal extraction time with methanol was studied. It was observed that

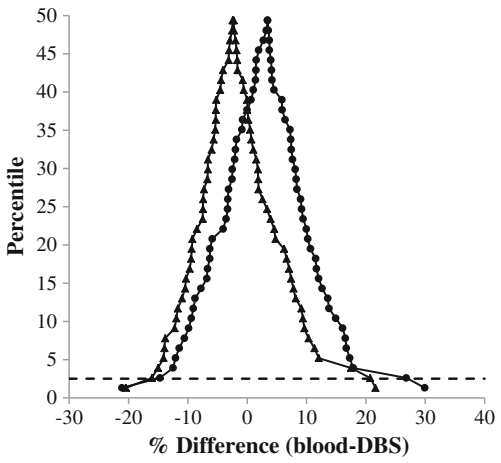

Fig. 2 Mountain plot of the percentage differences of EtG (*circles*) and EtS (*triangles*) between whole blood and DBS assays. The 2.5 or 97.5 percentile is represented in the figure by a *dashed line*

the extraction time could be reduced from 30 to 5 min still obtaining similar recovery for EtG and EtS.

As reported earlier [17, 36], the IS employed for quantification purposes were added to the elution solvent, even though it is regarded by some authors as not entirely satisfactory [26]. They claim that with this method the IS are not integrated into the sample prior to the extraction process and that therefore they might not correct for any variability during the extraction process [26]. However, employing other more laborious IS application techniques where the IS was integrated into the DBS samples prior to extraction showed to be only as good as the method employed in this study. The DBS method for the determination of EtG and EtS was successfully validated regarding linearity, accuracy and precision, selectivity, stability, and extraction efficiency.

The results of the matched-paired *t* test show that there is no significant difference between the two analytical methods in the determination of EtG and EtS. However, the Wilcoxon rank sum test indicates a significant difference between the two methods for both analytes. Even though this test is less meaningful than the *t* test due to the normal distribution of the differences, it points out that the differences are on the borderline between being significant and not significant.

This is confirmed by the Bland–Altman analysis and the Mountain plot. As it can be seen in Figs. 1 and 2, the whole blood and DBS method for EtG and EtS determination are biased with respect to each other. However, the bias calculated for EtG and EtS is 3.15 and −2.15 % (mean of the bias calculated with the Bland–Altman analysis and the Mountain plot), respectively, an acceptable error when measuring biological samples. The limits of agreement calculated with the Bland–Altman analysis and the Mountain plot show that the differences are between −18 and 27 % (mean of the limits of agreement calculated with the Bland–Altman analysis and the Mountain plot). However, as indicated in [34], the bias and large differences measured with these plots may have nothing to do with the analytical properties of the assay but with misidentified samples or other such mistakes. For this reason the matched-paired *t* test that takes into account possible outliers is the method of choice for method comparison. Most probably, duplicate analysis of the traffic offense case samples as DBS and elimination of outliers would give different results. However, the viability of EtG and EtS determination from DBS was demonstrated with these conditions, and the authors considered performing duplicate analysis or excluding outliers not necessary to proof this point.

Unlike DBS that are created with capillary blood obtained by skin puncture, the DBS specimens that were used in this study for method validation and comparison were created from heparinized venous whole blood samples, the so-called dried venous blood spots. Capillary blood differs from blood that is obtained through venipuncture and is usually contaminated with interstitial and intracellular fluids [37]. Due to the potential differences between venous and capillary blood analyte concentrations, the use of "dried capillary blood spots" should be preceded by a complete validation. The determination of EtG and EtS from capillary DBS would open up new possibilities in detecting recent alcohol consumption. The sample can be collected by people without medical background after an adequate training and in a controlled environment which avoids sample contamination, dilution, or sample mix-up. So, DBS analysis for EtG and EtS could be applied in fields like workplace monitoring, abstinence control, or drunken driving, where the time between offense and whole blood sampling would be rather long (several hours) and the ethanol could already be eliminated.

Conclusion

In this study, a new analytical method was presented for the quantification of two biomarkers of recent alcohol consumption by using DBS instead of liquid blood samples. EtG and EtS were analyzed in blood spots that were collected on filter paper and left to dry—the so-called DBS. The method was successfully validated according to the GTFCh guidelines, which largely coincide with internationally accepted guidelines. A comparison with the routinely employed whole blood method was performed. The statistical analysis revealed that both methods for EtG and EtS determination did not differ significantly, being the bias measured between whole blood and DBS irrelevant in toxicological analysis. Thus, the presented DBS method should be regarded as a promising method to determine EtG and EtS in blood, with the great potential of the method lying in the easy and inexpensive sample collection, transportation, and storage and the great stability of the biomarkers in DBS samples. In addition, phosphatidylethanol—an upcoming alcohol marker—can be analyzed from the same sample collection device [27, 28].

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