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Phosphatidylethanol compared to established indirect and direct markers of alcohol consumption

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1. Introduction

Misuse of alcohol involves a high risk of injuries, e.g. in road traffic, and diseases [1, 2]. Self-reporting being often of limited diagnostic efficiency and access to a detailed medical history being unavailable in particular settings, alcohol biomarkers may be an objective tool to assess alcohol exposure or misuse. The World Health Organisation in cooperation with the International Society on Biomedical Research on Alcoholism provided a comprehensive study on different markers such as carbohydrate deficient transferrin (CDT) and γ-glutamyltranspeptidase (GGT) activity [3-6]. Hartmann et al. [10] compared the direct alcohol consumption marker phosphatidylethanol (PEth) to GGT activity, CDT and the mean corpuscular volume (MCV). PEth is a phospholipid which is formed extrahepatically through phospholipase D in membranes of red blood cells if ethanol is present [7-9]. Further, yet established, direct markers are ethyl glucuronide (EtG) and ethyl sulfate (EtS) [11-14].

Dried blood spots (DBS) have routinely been used in neonatal screening and have recently been established as a valuable tool in therapeutic drug monitoring [15-17]. DBS are considered advantageous over whole blood specimens in many respects. Collection of DBS samples is easier, cheaper, less invasive and demanding, requiring a sample volume of 10-100 µL blood, only [18]. In addition, the risk of infections is reduced; e.g. HI- and hepatitis C viruses lose their infectivity in DBS, since drying leads to disruption of their envelope [19]. The DBS matrix has also proven to excellently stabilize 6-acetylmorphine, zopiclone and cocaine when compared to whole blood [20-22]. Therefore, DBS might offer a unique medium for measuring biomarkers that may be superior to traditional liquid specimens.

2. Aims

The aim of the present study was to determine major PEth species from DBS and corresponding whole blood samples taken from patients during alcohol detoxification treatment by LC-MS/MS to find out whether both media can be used interchangeably. Furthermore, stability of major PEth species in both matrices has been compared. In addition, measurement of EtG (serum, urine, hair), EtS (serum, urine) and CDT (serum) was included allowing to compare respective detection time and validity with regard to drinking patterns.

3. Material and Methods

The study protocol was approved by the local ethics committee and informed consent was obtained from each subject before enrolment in the study. Whole blood, serum, urine and hair samples were collected from 81 subjects in cooperation with the Psychiatric Center Nord-

baden (Wiesloch, Germany) and the Salem Medical Center (Heidelberg, Germany). Liquid specimens were collected on three different occasions whereas a hair sample was obtained once only. Corresponding blood spots were prepared by spotting 100 µL of venous blood onto a Whatman #903 filter paper (GE Healthcare) which was dried at room temperature for at least 3 h. Liquid specimens were stored at −80 °C until analysed whereas DBS and hair samples were kept at room temperature, respectively.

PEth, EtG and EtS were determined from whole blood, DBS, urine, serum and hair by LC-MS/MS following liquid/liquid extraction. Validation experiments were performed according to the GTFCh guideline [23]. For details on the determination of the two major PEth species PEth 18:1/18:1 and PEth 16:0/18:1 see reference [8]; a chromatogram of a DBS-sample having been prepared from an authentic specimen is shown in Fig. 1. An in-house method for the determination of EtG in urine has been modified to analyze EtG in hair.

In-house methods were used to analyse EtG and EtS in urine and serum as well as CDT in serum. Activity of GGT, aspartate (ASAT) and alanine (ALAT) transaminases was measured at the respective clinical facilities.

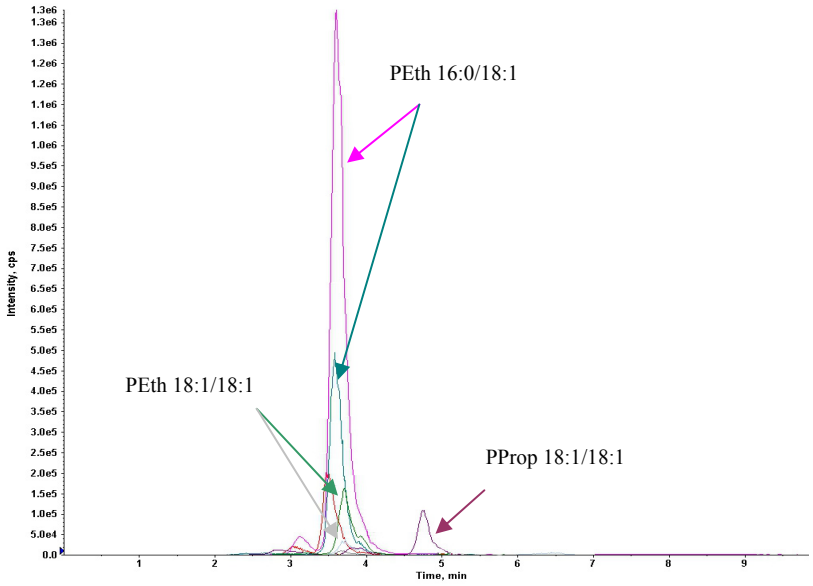

Fig. 1. Chromatogram of a dried blood spot prepared from an authentic whole blood specimen; PEth: phosphatidylethanol; PProp: phosphatidylpropanol (internal standard).

Exponential regression analysis allowed overcoming different time periods of collection; analyte concentrations thus could be assigned to day 0, 6 and 12. The paired student's t-test was used to compare the mean whole blood and DBS concentrations; further, blood/DBS ratios as well as their relative standard deviations (RSD) were calculated. In addition, linear regression, Bland-Altman difference plots, mountain-plots and the Passing-Bablok regression have been applied [24-26]. A possible relation between drinking patterns and markers was investigated using Box-Whisker plots and Kruskal-Wallis- and Wilcoxon-Mann-Whitney tests. The stability study, whose storage conditions are outlined in Tab. 1, comprised six trial arms:

1. Freshly drawn PEth free heparinized blood (10-30 U/mL heparin; Sarstedt, Germany) was either spiked (nominal values) with each 1000 ng/mL (low level) or 2000 ng/mL (high level) of both PEth species (PEth 18:1/18:1 and 16:0/18:1).
2. PEth free EDTA-blood (1.2 mg/mL K_3-EDTA) was spiked just as aforementioned (1.).
3. EDTA-blood samples (n=5, ethanol was not present by headspace/gas chromatography) from subjects undergoing alcohol detoxification treatment were pooled.

4. Aliquots of 100 µL of heparinized blood spiked (nominal values) with each 500 ng/mL (low level) or 1000 ng/mL (high level) of PEth 18:1/18:1 and, accordingly, 1500 ng/mL (low level) or 3000 ng/mL (high level) of PEth 16:0/18:1, were spotted onto Whatman #903 filter paper and dried at room temperature for 3 h.
5. Aliquots (100 µL) of spiked EDTA-blood (500 ng/mL or 1000 ng/mL PEth 18:1/18:1; 1500 ng/mL or 3000 ng/mL PEth 16:0/18:1) were spotted onto the filter paper and dried at room temperature for 3 h.
6. Aliquots (100 µL) of a second pool of authentic blood samples (n=5) from subjects undergoing alcohol detoxification were spotted onto Whatman #903 filter paper.

DBS were stored at 20°C and -20°C in zip-foil bags with a desiccant pack. One hundred µL aliquots of spiked and authentic whole blood were stored in 2 mL safe lock tubes (Eppendorf, Germany). All values are given as their mean (n=2) and as a percentage of the respective initial mean concentration.

Tab. 1. Time schedule for specimen processing and analysis; wb: whole blood; DBS: dried blood spot; -: no sample processing and analysis.

Day	-80°C	-20°C	4°C	20°C	40°C
0	wb	wb, DBS	wb	wb, DBS	wb
1	-	-	-	-	wb
2	-	DBS	wb	wb, DBS	wb
3	-	-	-	-	wb
4	-	DBS	wb	wb, DBS	wb
8	-	wb, DBS	wb	wb, DBS	-
21	wb	wb, DBS	wb	wb, DBS	-
30	wb	wb, DBS	wb	wb, DBS	-

A periodic analysis result that was within 15% of the initial concentration was used as the criterion of stability [27]. Rate constants at each storage temperature were determined to estimate an Arrhenius relationship and the activation energy assuming a monomolecular reaction.

4. Results

4.1. Validation data

Overall, validation parameters for the determination of the two PEth species in whole blood and DBS met the criteria of the GTFCh guideline showing a slight variance of matrix effect, extraction and process efficiencies (Tab. 2, validation of PEth 18:1/18:1, further data see [8].

4.2. Comparison of PEth analysis from whole blood and DBS

Results were normally distributed. The mean wb/DBS ratio of PEth 18:1/18:1 was 1.01 (range 0.87 – 1.57, RSD 8.22%). The mean difference (wb-DBS) was calculated at 0.38 ng/mL; the level of agreement was ± 9.38 ng/mL. The scatter plot revealed a regression coefficient of 1.0000. An evaluation of PEth analysis from blood and DBS for PEth 16:0/18:1 is summarized in Tab. 3. Figure 2 shows the Bland-Altman difference plot and the mountain plot for PEth 16:0/18:1.

Tab. 2. Validation results for the determination of PEth 18:1/18:1 from whole blood and DBS; LOD: limit of detection, LOQ: limit of quantification.

Validation parameter	Concentration [ng/mL]	Matrix	
		Whole blood	DBS
Linearity [ng/mL]		50-1,500	50-1,500
Calibration graph		y=1.00x-0.68	y=1.00x-3.33
Correlation coefficient R		0.9999	0.9959
LOD [ng/mL]		17.4	8.9
LOQ [ng/mL]		65.8	22.8
Inaccuracy [RSD %]	200/800	1.3/0.5	2.8/0.9
Within-run imprecision [RSD %]	200/800	11.5/10.4	9.7/6.9
Between-run imprecision [RSD %]	200/800	7.7/9.6	5.8/5.7
Matrix effect [%]	200/800	30.9/55.3	65.5/88.4
Extraction efficiency [%]	200/800	78.5/31.7	66.3/30.1
Process efficiency [%]	200/800	24.3/17.5	42.5/26.6

Tab. 3. Comparison of PEth 16:0/18:1 analysis from whole blood and DBS (n=81).

Test	Criterion	PEth 16:0/18:1
Mean wb/DBS ratio (RSD %)		0.99 (7.02 %)
t-Test	$mean_B = mean_{DBS}$	yes
Bland-Altman-Difference-Plot	mean difference	6.55 ng/mL
Mountain-Plot	median difference	0 ng/mL
Scatter Plot	regression coefficient	1.000
Passing-Bablok-Regression	95 % CI of slope includes 1.0	yes
	95 % CI of intercept includes 0.0	yes

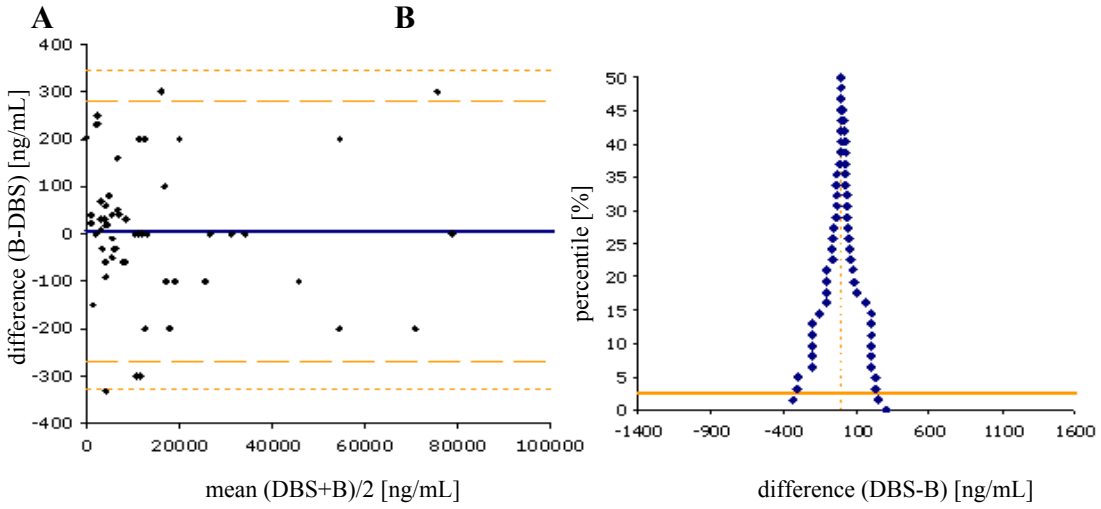

Fig. 2. Graphical method comparison of PEth 16:0/18:1 analysis in whole blood and DBS. A: Bland-Altman-Difference-plot: blue line: mean difference (B-DBS); orange dotted line: limit of agreement -267.55 and 280.88 ng/mL; fine dotted line: 95 % CI, ±336.23 ng/mL.

4.3. Stability of PEth in whole blood and DBS samples

A loss of >15 % of the initially measured concentration has been chosen as a criterion for instability instead of the critical difference [41]. Originally, the first criterion has been referred to the nominal concentration by Nowatzke and Woolf [27].

Stability of PEth species in heparinized blood samples. PEth 18:1/18:1 and 16:0/18:1 were stable for 30 days at a storage temperature of -80 °C. Both PEth species showed a significant reduction already after a one or two day storage when spiked whole blood was in blood at 40 °C, 20 °C and 4 °C. Nevertheless, about 80 % and 70 % of the initial concentration of PEth 18:1/18:1 and PEth 16:0/18:1, respectively, were still detectable after 30 days. The following activation energies could be estimated from the respective Arrhenius plot: PEth 18:1/18:1 38.9 kJ/mol (low level) and 37.4 kJ/mol (high level); PEth 16:0/18:1 31.8 kJ/mol (low level) and 36.7 kJ/mol (high level).

Stability of the analytes stored in EDTA-preserved blood. Both PEth species were stable for 30 days at a storage temperature of -80 °C. Both concentrations had declined by more than 15 % on day 21; nevertheless, 74.9 % (low level) and 78.6 % (high level) of the initial concentration of PEth 16:0/18:1 were detectable on day 30, and PEth 16:0/18:1 was stable for 8 days using the 15% criterion. The activation energy was estimated at 54.6 kJ/mol and 51.0 kJ/mol for the low and high concentration of PEth 18:1/18:1, and at 61.0 and 59.0 kJ/mol for PEth 16:0/18:1, respectively.

Stability of PEth species in authentic EDTA whole blood samples. PEth specimens in authentic blood samples showed a similar degradation pattern compared to that of spiked samples. PEth 18:1/18:1 was still stable on day 8 following storage at -20 °C; for PEth 16:0/18:1; a loss of >15 % was reached after 8 days of storage. Remaining concentrations of PEth 18:1/18:1 in samples stored at -20 °C and 20°C are given in Fig. 3. Activation energies were 102.4 kJ/mol for PEth 18:1/18:1 and 82.3 kJ/mol for PEth 16:0/18:1, respectively.

Stability of PEth 18:1/18:1 and 16:0/18:1 in spiked and authentic DBS samples. There was no significant decrease of PEth species prepared from spiked heparinized blood and stored for 30 days at -20°C at both concentrations. PEth (both species and concentrations) was stable for the whole observation period irrespective of the stability criterion applied. At room temperature more than 15 % of the initially measured concentration were lost after 30 days for PEth 18:1/18:1 (both levels) and PEth 16:0/18:1 (low level) in DBS prepared from EDTA blood whereas 93.7 % of the initially measured high PEth 16:0/18:1 concentration was still detectable after a storage of 30 days at 20 °C.

Both PEth species were stable in authentic DBS samples irrespective of the storage temperature (Fig. 3).

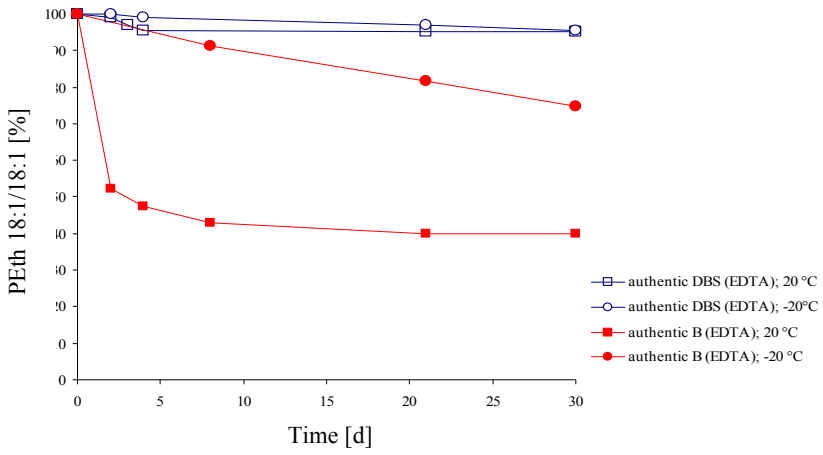

Fig. 3. Degradation of PEth 18:1/18:1 in authentic DBS (200 ng/mL) and whole blood (800 ng/mL, EDTA).

4.4. Indirect alcohol consumption markers

Interpolated mean values and concentration ranges of indirect markers on day 0, 6 and 12 are given in Table 4.

Tab. 4. Interpolated mean values and concentration ranges of carbohydrate-deficient-transferrin (CDT), γ-glutamyltranspeptidase (GGT), aspartate- (ASAT) and alanine (ALAT) transaminase activities on day 0, 6 and 12.

	Day 0	Day 6	Day 12
	CDT		
Mean value [%]	7.2	3.4	2.0
Concentration range [%]	0.5 – 70.1	0.5 – 13.3	0.2 – 11.9
	GGT		
Mean value [U/L]	451	320	271
Concentration range [U/L]	20 – 4,038	22 – 2,263	19 – 1,616
	ASAT		
Mean value [U/L]	128	61	34
Concentration range [U/L]	19 – 588	19 – 228	6 - 99
	ALAT		
Mean value [U/L]	90	61	48
Concentration range [U/L]	9 – 296	18 – 187	12 - 217

4.5. Determination of direct alcohol consumption markers

EtG was present in 19 out of 20 hair samples (> 2.5 pg EtG/mg hair, mean: 277 pg EtG/mg hair, range: 11 – 1,561 ng/mg hair). Results of EtG in urine and of PEth 18:1/18:1 and 16:0/18:1 in whole blood are summarized in Table 5.

Tab. 5. Interpolated mean values and concentration ranges of ethyl glucuronide (EtG) in urine and phosphatidylethanol (PEth) 18:1/18:1 and 16:0/18:1 in whole blood.

	Day 0	Day 6	Day 12
	EtG (urine)		
Mean value [ng/mL]	361,140	8,813	752
Concentration range [ng/mL]	125 – 7000,000	79 – 225,267	104 – 2,465
	PEth 18:1/18:1, whole blood		
Mean value [ng/mL]	563	170	128
Concentration range [ng/mL]	83 – 10,277	67 – 757	66 - 411
	PEth 16:0/18:1, whole blood		
Mean value [ng/mL]	17454	7746	4006
Concentration range [ng/mL]	191 – 143,061	130 – 44,429	89 – 36,470

4.6. Drinking pattern in relation to alcohol consumption markers

Subjects were classified as follows: 1: <600 g ethanol/week; group 2: ≥600 g ethanol/week. Data analysis was performed using Box-Whisker-plots of interpolated data (day 0). To test for a significant difference between median values, the Kruskal-Wallis test and the Wilcoxon-Mann-Whitney test were applied. Median values between group 1 and 2 did not significantly differ (CDT, EtG and EtS in urine, PEth).

5. Discussion

Comparison of PEth analysis from whole blood and DBS. The potential of DBS as an alternative medium for analysis of PEth species with PEth 16:0/18:1 and 18:1/18:1 being reportedly the predominant ones [28] has been shown. A prerequisite was successful downscaling of hitherto existing methods to 100 µL. Previously reported assays either determine only the sum of PEth-homologues [29] or apply 300 µL of whole blood for extraction [9]. Despite a reduced sample volume, we were able to determine the most abundant PEth species at a lower detection limit < 20 ng/mL. Overall, this sensitive assay may also allow detecting single drinking episodes or heavy drinking after a far longer time following abstinence when compared to other direct markers [30]. Differences between the two methods (DBS, whole blood) were rather evenly distributed across the concentration range. Statistics revealed that analysis of PEth species from DBS is as reliable as that from traditional specimens.

Stability of PEth in whole blood and DBS samples. There is no doubt that PEth species are unstable analytes, and that blood samples should preferably be stored at -80 °C. Stability results obtained from spiked samples are in line with previously published data from Helander and Zheng [31] where PEth in whole blood stored at -80 °C was stable for a period of 14 months. Aradottir et al. [7] reported on PEth in snap-frozen whole blood samples being stable for 6 days without giving the storage temperature.

DBS appears to be suitable matrix to stabilize PEth species at conditions that are commonly available in a forensic laboratory. In addition, the anticoagulant did not influence the stability of PEth in DBS in contrast to the results obtained from whole blood specimens.

Interestingly, the activation energy of EDTA samples was higher than of heparinised blood samples suggesting an influence of the anticoagulant on the stability of PEth species in whole blood. EDTA can prevent oxidation of lipids by trace metal ions during processing of blood samples [32]; and inhibition of phospholipase due to depletion of calcium ions may reduce hydrolysis of phospholipids [33]. PEth species being still bound within or attached to the erythrocyte membrane are considered less susceptible to degradation. This may be an explanation why authentic EDTA blood samples showed a slightly higher stability compared to spiked EDTA containing specimens. Also, stability of thawed samples may be decreased over freshly drawn samples [36-38].

Activity of enzymes in whole blood samples, chemical hydrolysis and oxidation are processes responsible for analyte degradation [34, 35]. Degradation pattern of PEth species suggests that the main underlying mechanisms may be hydrolysis. A possible oxidation of the double bonds of PEth 18:1/18:1 and 16:0/18:1 has been considered minor or insignificant by Bell et al. [39] and Le Grandois et al. [40]. 5.3 Drinking pattern in relation to alcohol consumption markers

Box-Whisker-plots of CDT revealed no difference between the 2 groups. A likely explanation can be that assessing of the subjects' drinking behaviour from an objective point of view is rather difficult. On the other hand, all patients included in the study had a history of alcohol misuse, at least. An increase of CDT is already detectable if alcohol consumption comes close to 50-80 g ethanol/die over 7-10 days [42, 43]. The observed CDT concentrations are in line with data published by Wurst et al. [44], Tabakoff et al. [45] and Helander et al. [4]. The plots of EtG and EtS determined from urine suggest a difference between the median values of both groups which, however, did not reach statistical significance. The very rapid decline of EtG- und EtG-concentrations following abstinence from alcohol may serve as an explanation. In addition, self-reporting may have been limited.

Evaluation of PEth 18:1/18:1 and PEth 16:0/18:1 revealed no statistical difference between both groups which may be due to the broad range of values as well as to the slow decline after cessation of alcohol consumption.

6. Conclusions

CDT proved to be a very specific marker to detect alcohol misuse whereas activities of GGT, ASAT and ALAT as well as MCV should not at all be applied in isolation.

Testing for EtG and EtS in urine is clearly superior to analysis of serum for monitoring alcohol use. Unsurprisingly, a longer period of time can be followed using hair for testing EtG. Even low doses of alcohol are detectable in hair and urine.

According to the results for PEth species, a detection window of more than 12 days will allow a reliable means to monitor abstinence from alcohol. At present, this marker is suggested to be superior to hitherto established markers of alcohol intake. At present, it is not quite certain whether formation of PEth species will depend on extraneous influences. In addition, an appropriate choice of PEth species for routine analysis and establishment of a cut-off value to differentiate social drinking from alcohol misuse are considered major research objectives in the future.

DBS appears suitable to stabilize PEth compared to conventional blood samples offering numerous advantages such as ease of storage and transportation, sensitive, accurate and precise analysis and absence of post collection synthesis. At present, DBS can be stored at room temperature for 30 days without affecting stability of PEth. Further studies are necessary to test whether an extended period of time will affect PEth's stability.

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