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Technical Note

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Stability of Phosphatidylethanol 16:0/18:1 in Freshly Drawn, Authentic Samples from Healthy Volunteers

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Abstract

Due to its specificity, phosphatidylethanol (PEth) 16:0/18:1 has gained increased popularity as a marker for high alcohol consumption in recent years. As conflicting results regarding the stability of PEth 16:0/18:1 in whole blood have been published, there are still uncertainties related to to optimum handling, transport and storage of blood samples for the analysis of PEth 16:0/18:1. A stability study where whole blood samples were drawn from healthy volunteers, who had ingested alcohol, is presented. The samples were collected in tubes with ethylenediamine tetraacetic acid (EDTA) and heparin as additives and stored under standardized conditions within 1 h of blood sampling. Storage times were 28 days in ambient temperature and at 4–8°C, and 90 days at −20°C and −80°C. All samples were analyzed regularly during the storage periods. PEth 16:0/18:1 concentrations were stable (defined as < 15% decrease compared with baseline values) at all temperatures up to 28 days, independent of additive. After 90 days of storage at −20°C, the mean concentrations had decreased by 18.8% in EDTA tubes and by 13.8% in heparin tubes. At −80°C, the concentrations were stable throughout the 90-day period. The present study shows that in samples containing PEth formed *in vivo*, PEth 16:0/18:1 is stable for 28 days irrespective of storage temperature. During long-term storage, samples should be stored at −80°C.

Introduction

Phosphatidylethanol (PEth) is a collective term for abnormal phospholipids formed from fatty acids in the membranes of various cell types including red blood cells, in the presence of ethanol via the action of phospholipase D (1, 2). Almost 50 different homologs of PEth have been identified, with 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphoethanol (PEth 16:0/18:1) as one of the most abundant (3–7). Because PEth is formed exclusively in the presence of ethanol, it has been increasingly used as a marker for alcohol intake. Due to its specificity and because it has a detection window of up to several weeks after alcohol intake, its application potential is broader than for other ethanol biomarkers (8).

Very often the sampling site and the analytical laboratory are located separately, and consequently, sample transportation to the analytical laboratory, occasionally even by mail, is required. Through this process, the samples will have a certain storage time before analysis, and during this time, the temperature conditions will vary. In addition, the samples will routinely be stored at the laboratory after analysis for some time in case of re-analysis. It is obvious that if storage conditions affect the stability of PEth, the validity of the analytical result is compromised. To ensure the validity of

an analytical result, it is therefore a prerequisite to have detailed knowledge of the stability of the analyte under various storage conditions.

Some studies addressing the stability of PEth both in whole blood and in dry blood spots have been published (6, 9–12). PEth is found to be stable when stored at −80°C or colder, probably for as long as 20 years (6, 10–12). Helander et al. (6) reported that PEth was stable in whole blood for at least 5 days at 4°C. Aradottir and Olsson (9) reported that PEth was stable in whole blood for at least 3 weeks when kept refrigerated. In the latter study, it was also investigated whether the anticoagulant added into the sample tube affected stability, and no significant differences between EDTA and heparin blood were found. In contrast, Faller et al. (12) found that PEth 16:0/18:1 had a temperature-dependent instability in whole blood during storage: only 49% of the initial concentration was left after 2 days of storage at 20°C in EDTA blood. At −20°C, PEth 16:0/18:1 was more stable, but after 30 days the concentration was reduced to 75%. Moreover, the type of anticoagulant seemed to affect the stability, as PEth was even less stable in heparinized blood than in EDTA blood. It should, however, be emphasized that these findings are based upon spiked samples. The authors briefly state that 'PEth specimens in authentic blood samples showed a similar degradation pattern compared with that of spiked samples' and 'for PEth 16:0/18:1, a loss of >15% was reached after eight days of storage'. All factors affecting the degradation rate of PEth *in vitro* is not known, but it is possible that PEth incorporated in the cell membrane degrades slower than PEth molecules freely dissolved in the vial (12), e.g. due to a lower availability for enzymatic or non-enzymatic breakdown for cell membrane PEth compared with plasma PEth. This may explain why PEth 16:0/18:1 degraded faster in the spiked samples than in the authentic samples.

In a previous study (13), we examined the stability of PEth 16:0/18:1 in patient samples with EDTA after they had been sent to our laboratory by mail or courier. Using a 15% decrease as cut-off, the samples were stable at 30°C and 4–8°C for least 5 days and 4 weeks, respectively, nor were there any signs of degradation in patient samples after three freeze (−80°C) and thaw cycles (13). In the study by Faller et al. (12), most of the reduction in the PEth 16:0/18:1 concentration occurred during the first 2 days of observation. One may therefore question whether the PEth 16:0/18:1 concentration in our previous study (13) already had dropped during transport, before the baseline analysis and that we therefore were not able to observe any further reduction. The aim of the present study was to evaluate the stability of PEth 16:0/18:1 in authentic samples where standardized storage conditions were established immediately after sampling.

Methods

The authentic samples were collected at the Clinical Research Unit at St. Olavs University Hospital in Trondheim, Norway. Ten healthy volunteers, 5 women and 5 men, were recruited. All subjects gave their informed written consent before sampling. They were at least 18 years old (age of majority in Norway). To ensure that the level of PEth in the blood would be sufficient to quantify a possible degradation of PEth, we only included volunteers who had consumed at least 10 units of alcohol per week the last 2 weeks before sampling (1 unit of alcohol was defined as 13 g of ethanol). Their mean age was 23 years (range 20–27 years).

In total, 50 mL of whole blood was drawn from each subject, half on EDTA tubes (Vacuette K2 EDTA, 6 mL; Greiner Bio-One GmbH, Austria) and half on sodium heparin tubes (Vacuette NH Sodium Heparin 4 mL, Greiner Bio-One GmbH, Austria). The samples were partitioned into 0.6-mL aliquots and stored under standardized conditions within 1 h after sampling.

Within 1 h after sampling, the laboratory received three aliquots of blood with heparin and three with EDTA from each subject, and sample preparation and analysis were immediately carried out. Mean concentration of these three samples was used as the baseline value for each additive. The samples were frozen to −80°C for ∼30 min to hemolyze the red blood cells. One sample from each donor was also analyzed for ethanol with our validated and accredited headspace gas chromatography–flame ionization detector (GC–FID) method routinely in use for alcohol analysis.

Storage conditions

The aliquots were stored at ambient temperature (mean 22.1°C, range from 20.7 to 23.6°C), 4–8°C, −20°C or −80°C. One aliquot with each additive at each temperature was stored and analyzed. The aliquots were stored for up to 28 days in ambient temperature and 4–8°C, and at −20°C and −80°C for up to 90 days.

Analytical method

PEth 16:0/18:1 was analyzed with a validated ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS-MS) method described in detail previously (13). In brief, blood samples stored at −20°C or −80°C were thawed to room temperature before sample extraction. The blood samples stored at 4–8°C and at ambient temperature were frozen to −80°C for haemolysis to take place followed by thawing to ambient temperature, before sample extraction.

A 150-µL sample was protein-precipitated with 2-propanol (450 µL) containing the internal standard (PEth 16:0/18:1-d_5, 0.55 µM), then mixed and centrifuged before 2.0 µL of the supernatant (0.075 µM on column for the lowest calibrator) was directly injected into the UPLC–MS-MS system (Xevo TQS, Waters, Milford, MA, USA). Chromatographic separation was achieved with a Waters Acquity BEH-Phenyl column (2.1 × 30 mm, 1.7 µM) with pre-column using a gradient elution starting with 40% ammonium formate (5 mM, pH 10.1, mobile phase A) in combination with 60% acetonitrile (mobile phase B). During the first 90 sec the gradient went from 60 to 95% B, then kept with 95% B for 6 sec. A total of 42 sec with 60% B were found sufficient to equilibrate the column before next injection. The flow rate was 0.5 mL/min and the runtime 2.3 min.

A 150-µL sample was protein-precipitated with 2-propanol (450 µL) containing the internal standard (PEth 16:0/18:1-d_5, 0.55 µM), then mixed and centrifuged before 2.0 µL of the supernatant (0.075 µM on column for the lowest calibrator) was directly injected into the UPLC–MS-MS system (Xevo TQS, Waters, Milford, MA, USA) without filtration. Chromatographic separation was achieved with a Waters Acquity BEH-Phenyl column (2.1 × 30 mm, 1.7 µM) with pre-column, using a gradient elution starting with 40% ammonium formate (5 mM, pH 10.1, mobile phase A) in combination with 60% acetonitrile (mobile phase B). During the first 90 sec the gradient went from 60% to 95% B, then kept with 95% B for 6 sec. A total of 42 sec with 60% B was found sufficient to equilibrate the column before next injection. The flow

Table I. PEth 16:0/18:1 Concentrations in the Samples Analyzed Within 1 h after Sampling[a]

Subject number	PEth concentration, EDTA as additive µM (SD)	PEth concentration, heparin as additive, µM (SD)	Relative difference in PEth concentration, heparin compared to EDTA, %
1	0.309 (0.006)	0.301 (0.005)	−2.8
2	0.212 (0.002)	0.185 (0.004)	−12.6
3	0.053 (0.003)	0.046 (0.003)	−13.3
4	0.109 (0.001)	0.098 (0.001)	−10.4
5	0.082 (0.001)	0.066 (0.002)	−10.0
6	0.597 (0.010)	0.523 (0.007)	−12.4
7	0.080 (0.002)	0.072 (0.002)	−19.1
8	0.051 (0.001)	0.047 (0.002)	−7.8
9	0.156 (0.003)	0.138 (0.019)	−11.7
10	0.056 (0.002)	0.047 (0.002)	−15.5
Mean (SD)	0.171 (0.003)	0.152 (0.004)	−11.6 (4.4)

[a]Numbers represent the mean concentrations of three separate runs with standard deviations (SDs).

rate was 0.5 mL/min, and the runtime was 2.3 min. For detection and quantification of PEth 16:0/18:1 in negative ionization mode the *m/z* 701.7 > 255.2 and *m/z* 701.7 > 281.3 (*m/z* 706.7 > 281.3 for PEth 16:0/18:1-d_5) transitions were used. Calibration range of the method was 0.030–4.00 µM, and the within- and between-assay precisions were in the range of 0.4–3.3% (coefficient of variation ≤ 7.1%).

The ethanol analysis was carried out using a headspace GC–FID system (GC Trace 1310 with TriPlus RSH, Thermo Fisher Scientific, Milano, Italy). In brief, a 100-µL sample was mixed with 1000 µL internal standard (1-propanol, 0.015 mg/mL) and incubated for 20 min at 50°C before analysis. A 1.0-mL headspace sample was drawn with a pre-heated (85°C) syringe and injected (inlet temperature 250°C) into the GC–FID system (split flow 150 mL/min, split 30). Chromatographic separation was achieved using a Restek Rtx BAC Plus1 column (30 m × 0.32 mm × 1.8 µM, Restek, Bellefonte, PA, USA) with an isocratic column oven temperature of 45°C and a helium carrier gas flow of 5.0 mL/min. The FID was set to 275°C and the runtime was 3.6 min.

Data analysis

Data were analyzed with IBM SPSS Statistics v25 (IBM, Armonk NY, USA) and Microsoft EXCEL 2016 (Microsoft Corporation, Redmond, WA, USA). A decrease in mean concentrations of <15% from Day 0 was predefined as stability (14).

Results

The PEth 16:0/18:1 concentrations in samples analyzed on the day of sampling (Day 0) ranged from 0.05 to 0.60 µM (mean 0.17 µM) in EDTA samples and from 0.05 to 0.52 µM (mean 0.15 µM) in heparin samples (Table I). Ethanol was not detected in any of the samples.

Stability in EDTA samples

The mean deviation of PEth 16:0/18:1 concentration in percent from Day 0 is presented in Figure 1 and Supplementary Table SI. The mean values of samples stored at room temperature, 4–8 and at −80°C fulfilled the stability criteria throughout the observation period (up to 28 days for room temperature and 4–8°C and up to 90 days for samples stored at −80°C). For the EDTA samples stored at −20°C for 90 days, the mean PEth16:0/18:1 concentration was 18.8% lower than baseline, and these samples thus did not meet the predefined stability criteria.

Stability in heparin samples

The mean deviation of PEth 16:0/18:1 concentration in percent from Day 0 is presented in Figure 1 and Supplementary Table SI. The mean values of samples stored in room temperature and at 4–8, −20 and −80°C fulfilled the stability criteria throughout the observation period, although it should be noted that the mean concentration was 13.8% lower than Day 0 after 90 days of storage at −20°C.

Discussion

In our study, PEth 16:0/18:1 was stable for 28 days at all temperatures, irrespective of additive. After storage at −20°C for 90 days, the concentrations had fallen below the predefined limit of stability in the EDTA samples and were close to that limit also in the heparin samples. At −80°C, PEth 16:0/18:1 was stable both in the EDTA and the heparin samples throughout the period of 90 days.

The results of this study implicate that samples for analysis of PEth 16:0/18:1 can be sent by ordinary mail, including in sparsely populated areas and over long distances with prolonged transport time between sample site and laboratory. Samples can also be stored refrigerated or even in room temperature for a possible re-analysis for up to 28 days. For long-term storage, the samples must be kept at −80°C.

Our findings are in line with most previously published studies, although they differ from the results published by Faller et al. (12). Nevertheless, our results are in accordance with theory proposed by Faller et al. (12) that PEth species that are still bound within, or are attached to, the red blood cell membrane are less susceptible to hydrolysis than molecules present in the fluid compartment of blood, and that PEth in spiked samples therefore is more unstable than PEth in naturalistic samples. As our samples were handled as regular clinical samples at our laboratory, except for the short time between sampling and storage at the standardized conditions, we have no reason to believe that ordinary clinical samples should not be just as stable as these test samples.

In the present study, the stability criteria were met in samples stored for 90 days at −20°C when heparin was used as anticoagulant, but not when EDTA was used. We think this is an incidental finding

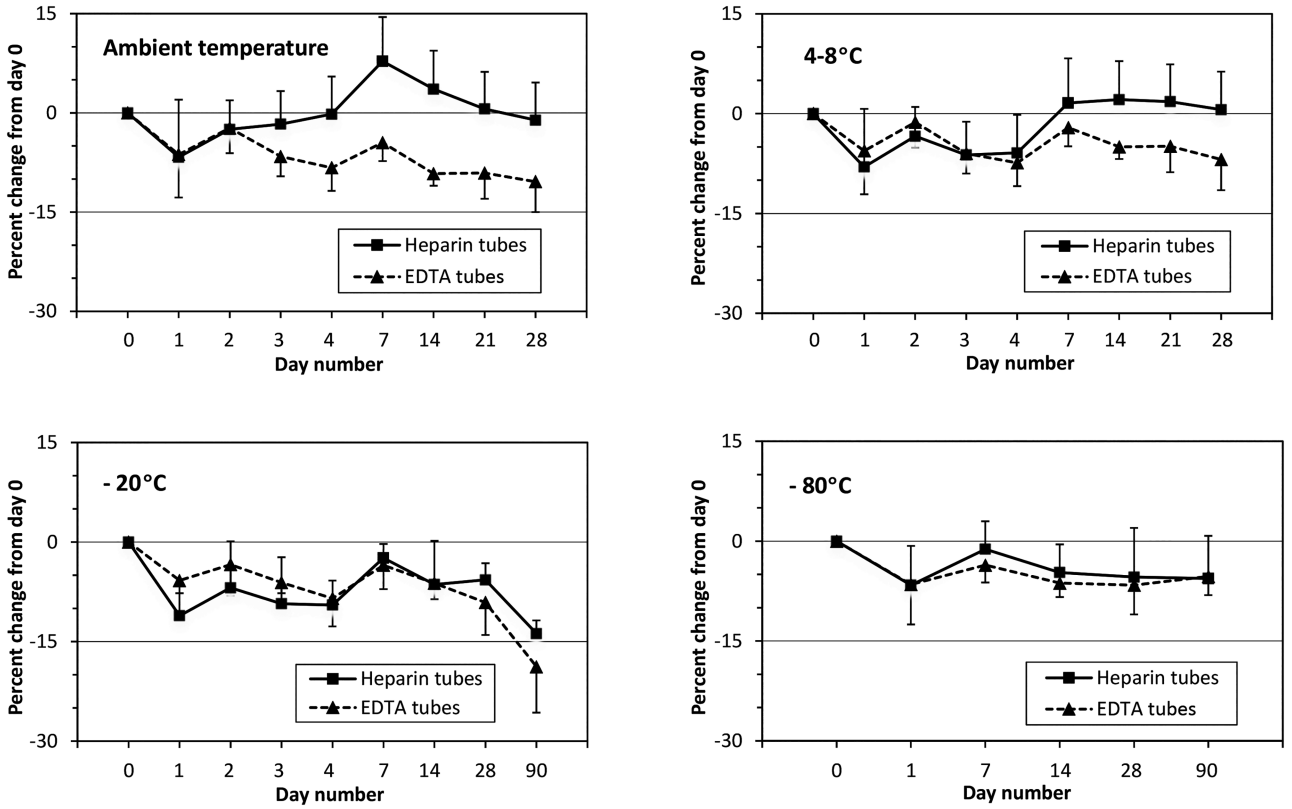

Figure 1. Percent change in PEth 16:0/18:1 concentrations compared with Day 0 after storage in EDTA tubes and heparin tubes at ambient temperature, 4–8°C, −20°C and − 80°C, respectively. Data are presented as means of the analyses from 10 healthy volunteers. Error bars represent relative standard deviations.

as also the PEth 16:0/18:1 concentration in the heparin samples showed a clear decreasing trend during the 90-day storage. Although recommended in the literature (14), the predefined 15% limit for defining instability is rather arbitrarily, and if the limit had been set to 12 or 10%, samples with both additives had been defined as instable. A more rapid decrease in the EDTA samples is apparently opposite to the findings of Faller et al. (12). However, with a few exceptions the differences are numerically small, and a closer look both at our results and at the findings of Faller et al. may indicate a pattern where PEth is more stable in EDTA tubes than in heparin tubes during the first days or so after sampling. Over a longer time perspective, this difference may tend to disappear, and in some cases, such as for samples stored at ambient temperature in our study and at 20°C in the study by Faller et al., there were even indications of higher long-term degradation of PEth in EDTA tubes than in heparin tubes. The lower initial degradation in EDTA tubes may be related to the fact that the anticoagulant effect of EDTA is based on chelation of calcium ions. A decrease in the levels of transition metals such as iron and copper is also reported (15). Thus, EDTA may reduce both the effect of possibly calcium-dependent enzymes involved in the degradation of PEth, as well as oxidation of lipids involving trace metal ions (16).

In samples containing ethanol, PEth can also be produced *in vitro* after sampling, as the enzyme phospholipase D is still active, even at low temperatures (17). Thus, in a sample containing ethanol the PEth concentration measured after storage could be affected both by *in vitro* production of PEth and degradation of PEth. In the present study, all samples were documented not to contain ethanol and therefore, our results represent degradation, only. In order to avoid a falsely high PEth values, the best sampling strategy in a subject under the influence of alcohol would be to postpone sampling until the blood alcohol concentration has decreased to zero.

The strength of this study is that pre-analytical conditions were controlled as thoroughly as possible, since all samples were stored at the correct temperature within 1 h of sampling. This study is also performed using authentic samples, that is, in samples where PEth 16:0/18:1 is formed *in vivo* and not added *in vitro*. It is a limitation that the concentration range of PEth 16:0/18:1 in the 10 samples included was quite narrow, with no samples with high concentrations. Therefore, we do not know whether the degree of instability will be constant irrespective of the concentration. However, we have no reason to believe that there should be any differences, as we did not identify any concentration-dependent variation within the concentration range we were able to evaluate.

Conclusion

The present study demonstrates that PEth 16:0/18:1 formed *in vivo* is stable for 28 days, irrespective of storage temperature. During long-term storage the samples should be kept at −80°C.

Supplementary data

Supplementary data is available at *Journal of Analytical Toxicology* online.

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