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Fully Automated Determination of Phosphatidylethanol 16:0/18:1 and 16:0/18:2 in Dried Blood Spots

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Abstract

Purpose: Direct alcohol markers are widely applied during abstinence monitoring, driving aptitude assessments and workplace drug testing. The most promising direct alcohol marker was found to be phosphatidylethanol (PEth). Compared to other markers it shows a long window of detection due to accumulation in blood. To facilitate and accelerate the determination of PEth in DBS, we developed a fully automated analysis approach.

Methods: The validated and novel online-SPE-LC-MS/MS method with automated sample preparation using a CAMAG DBS-MS 500 system reduces manual sample preparation to an absolute minimum, only requiring calibration and quality control DBS.

Results: During the validation process, the method showed a high extraction efficiency (>88%), linearity (correlation coefficient >0.9953), accuracy and precision (within ±15%) for the determination of PEth 16:0/18:1 and PEth 16:0/18:2. Within a run time of about 7 min, the two monitored analogs could be baseline separated. A method comparison in liquid whole blood of 28 authentic samples from alcohol use disorder patients showed a mean deviation of less than 2% and a correlation coefficient of >0.9759. The comparison with manual DBS extraction showed a mean deviation of less than 8% and a correlation coefficient of >0.9666.

Conclusions: The automated analysis of PEth in DBS can provide a fast and accurate solution for abstinence monitoring. In contrast to the manual extraction of PEth in DBS, no laborious sample preparation is required with this automated approach. Furthermore, the application of the internal standard by a spray module can compensate for extraction bias and matrix effects.

Introduction

A simple method to monitor alcohol abstinence is the determination of ethyl glucuronide (EtG) and ethyl sulfate (EtS) in urine. Due to the short window of detection, these two markers require frequent sampling. This results in a large number of liquid urine samples which need to be transported and stored before analysis (1). The discovery of phosphatidylethanol (PEth) as a direct alcohol marker extended the window of detection for alcohol consumption to several weeks. Due to the fast formation rate and slow degradation, it accumulates in cell membranes at about 1–2% of the total cellular phospholipid pool (2, 3). PEth allows one to distinguish heavy from occasional drinkers and to monitor alcohol abstinence (4–6).

PEth is present as a group of abnormal phospholipids with a polar phosphoethanol head. Fatty acid chains are attached at positions sn-1 and sn-2. Up to 48 different PEth analogs have been detected with various combinations of fatty acyl chain lengths and numbers of double bonds. The predominant species in blood after alcohol consumption are PEth 16:0/18:1 (30–46%) and PEth 16:0/18:2 (16–28%) (7–12). PEth in whole blood proved to be unsuitable for routine analysis, as it is unstable during storage and transportation above −80°C. Furthermore, PEth may be generated *in vitro*, post-sampling, if ethanol is present. This can lead to false positive results (10, 13).

By using dried blood spot (DBS) sampling, PEth can be stabilized due to the inactivation of enzymatic activity. Sampling as DBS

prevents post-sampling formation of PEth, allows shipping without biohazard labeling in a standard envelope and does not require any sample cooling. Several DBS methods for monitoring PEth have been developed, showing the feasibility of PEth determination in the dried format. The protocols involve manual punching of DBS cards and long sample preparation steps, including incubation, centrifugation and sonification (14–16).

The aim of this study was to develop a method for the fully automated sample preparation, extraction and online LC-MS/MS quantification for the two most abundant PEth species: PEth 16:0/18:1 and PEth 16:0/18:2. The main focus was thereby put on a system offering high-throughput and minimal turnaround time. Automation for this study was implemented by the CAMAG DBS-MS 500 extraction system and a triple quadrupole mass spectrometer coupled to an HPLC system.

Experimental

Chemicals and reagents

The internal standards for the PEth determination (16:0/18:1-D_5 and PEth 16:0/18:2-D_5) were synthesized as described elsewhere (17). The internal standard solution was prepared by mixing both analogs with 2-propanol.

Ammonium acetate, fractopur and methanol, with a purity of Reag. Ph Eur (European Pharmacopoeia), were obtained from Merck (Darmstadt, Germany). 2-Propanol, HPLC grade, was purchased from Fisher Chemical (Reinach, Switzerland). Acetonitrile, p. a., was ordered from Acros Organics (Geel, Belgium). Certified spiking solution for phosphatidylethanol 16:0/18:1 and 16:0/18:2 were purchased from Cerilliant (Round Rock, USA) and Avanti Polar Lipids, Inc. (Alabaster, USA). Formic acid, puriss p.a. 98%, was purchased from Sigma-Aldrich (Buchs, Switzerland). De-ionized water was produced with a Milli-Q water system from Millipore (Billerica, USA). BioSample TFN filter paper DBS cards from Ahlstrom (supplied by CAMAG, Switzerland) were used to produce the volumetric DBS.

Calibrator and quality control samples

For the quantification of PEth, a six-point calibration was prepared at 20, 75, 188, 375, 750 and 1,500 ng/mL (K1–K6). For the verification of the calibration curve, three quality control samples were prepared at 20, 45 and 1,180 ng/mL (QC1–QC3). To prepare the samples, 240 µL of blank blood from a teetotaller (containing no PEth) was spiked with 10 µL of stock solution containing PEth 16:0/18:1 and PEth 16:0/18:2 in 2-propanol. Subsequently, the spiked samples were mixed for 4 h. Afterwards, DBS spots containing 20 µL of blood were prepared on DBS cards. Before the extraction, the cards were dried for three hours. Afterwards, they were stored in a mingrip bag together with a silica gel pack and stored at −20°C.

Batch design and QC strategy

To provide a reliable measurement and to monitor the robustness of the automated system, each batch of analysis contained at least one set of six calibrator samples (K1–K6), a quality control sample at each concentration (QC1–QC3), negative controls (with and without internal standard added) and a blank (card without any blood added).

Fully automated sample extraction

The DBS-MS 500 system (CAMAG, Switzerland) was attached as front end to the online-SPE-LC-MS/MS system. The extraction solvent for the DBS elution consisted of water, acetonitrile, 2-propanol and formic acid (34.5:15:50:0.05, v/v/v/v). Each DBS was photographed with the built-in camera prior and after each run to check for the presence of a spot, to adjust the extraction head and to verify that an extraction took place. Internal standard solution (20 µL) containing the deuterated PEth analogs was applied by a spray head in a homogenous layer onto each spot. In contrast to spiking the internal standard into the sample tube, this procedure enables to compensate for extraction differences (recovery bias) (18). Afterwards, the card was dried for 30 s, before the extraction of a 4.2 mm sub-punch with a volume of 40 µL at a flow rate of 40 µL/min took place. After the extraction process, the extraction head and outlet were rinsed with the inbuilt wash station three times, each step taking 20 s. Rinsing solution 1 (85% 2-propanol, 15% water, v/v, containing 13 mM ammonium acetate), rinsing solution 2 (50% water, 50% acetonitrile, v/v) and rinsing solution 3 (2-propanol).

Manual DBS extraction

Spots were generated by adding 20 µL of blood on DBS cards. To compensate for a potential extraction bias, the internal standard was sprayed with the CAMAG DBS-MS 500 autosampler. Manual extraction of the complete DBS spot (20 µL) was performed by using 1 mL of methanol and subsequently shaking the sample for 4 h on a Vortex-Genie 2 (Scientific Industries Inc., New York, USA). Afterwards, centrifugation for 10 min at 16,000 g took place (Mikro 220 R, Hettich, Switzerland). The supernatant was transferred into champagne-cup glass vials, evaporated to dryness in a heating block set at 50°C, with a gentle stream of nitrogen, and reconstituted in 100 µL of elution solvent (water, acetonitrile, 2-propanol and formic acid (34.5:15:50:0.05, v/v/v/v)).

Manual whole blood extraction

To determine PEth in whole blood, 250 µL of blood was mixed with 100 µL 2-propanol and vortexed. Afterwards, 150 µL 2-propanol and 750 µL acetonitrile were added. The sample was vortexed again, and shaken for 45 min, before centrifugation for 10 min at 16,000 g was performed. The supernatant was transferred into glass vials, evaporated to dryness in a heating block set at 50°C, with a gentle stream of nitrogen, and reconstituted in 800 µL of elution solvent (water, acetonitrile, 2-propanol and formic acid (34.5:15:50:0.05, v/v/v/v)).

Online SPE-LC-MS/MS analysis

The online SPE-LC-MS/MS system consisted of an UltiMate® 3000 UHPLC system (Dionex, Thermo Scientific Instruments, Reinach, Switzerland) coupled to a Sciex 5500 QTRAP (Toronto, Canada) operated in negative SRM mode at −4,500 V, see Figure 1 and Table I. At first, the analytes were trapped on a polar-RP column, 20 mm × 2 mm, 4 µm particle size (Phenomenex, Brechbühler, Schlieren, Switzerland) which was preconditioned with A1 (water containing 0.1% formic acid v/v). Substances not interacting with the column were directed to the waste container. After 0.25 min elution with 100% B1 (70% acetonitrile, 30% water, v/v, containing 2 mM ammonium acetate) started during 2.25 min, whereby the ten-port valve at the UHPLC system was switched from the waste to the analytical column at 0.8 min. As analytical column, a Luna RP-C5 column, 50 mm × 2 mm, 5 µm particle size (Phenomenex, Brechbühler, Schlieren, Switzerland) preheated to 60°C was used.

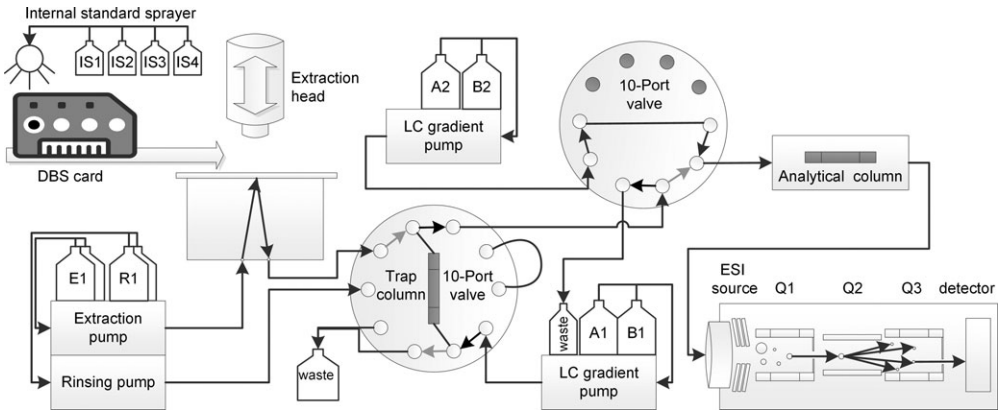

Figure 1. Instrumental setup for the fully automated analysis of DBS samples. The internal standard is sprayed onto the DBS, followed by extraction and concentration on a trapping column by the DBS-MS-500 system. Chromatographic separation is achieved by liquid chromatography, before the detection by tandem mass spectrometry is performed. Black arrows indicate the loading phase, gray arrows indicate the elution phase.

Table I. Parameters used for the selected reaction monitoring (SRM) of PEth 16:0/18:1 and PEth 16:0/18:2

Compound	Q1 [*m/z*]	Q3 [*m/z*]	Dwell time [ms]	DP [V]	EP [V]	CE [V]	CXP [V]	RT [min]
PEth 16:0/18:1 SRM 1	701.3	255.2	20	−32	−10	−40	−14	3.53
PEth 16:0/18:1 SRM 2	701.3	281.3	20	−20	−10	−40	−14	
PEth 16:0/18:1–D_5 SRM 1	706.3	255.3	20	−20	−10	−40	−14	
PEth 16:0/18:1–D_5 SRM 2	706.3	281.1	20	−32	−10	−40	−14	
PEth 16:0/18:2 SRM 1	699.5	279.4	20	−5	−10	−40	−14	3.29
PEth 16:0/18:2 SRM 2	699.5	255.3	20	−5	−10	−40	−14	
PEth 16:0/18:2–D_5 SRM 1	704.5	279.5	20	−5	−10	−40	−14	
PEth 16:0/18:2–D_5 SRM 2	704.5	255.3	20	−5	−10	−40	−14	

An inline filter (KrudKatcher Ultra, Phenomenex) was connected upstream to the analytical column. The analytical column was preconditioned with A2 (30% water, 70% acetonitrile, v/v, containing 0.6 mM ammonium acetate). The valve was switched back to the initial position at 1.7 min. Afterwards, the gradient elution with B2 (100% 2-propanol) started: The gradient for the analytical column was as follows: Start at 0% B2, followed by an increase to 25% B2 from 2.0 to 4.0 min. Afterwards, 100% B2 from 4.1 to 5.0 min. The flow rate for all pumps was set to 0.5 mL/min. The total run time was 5 min. For a chromatogram, see Figure 2.

Results

Linearity

Linearity was established during three validation series, whereby the calibrators K1–K6 were measured in duplicate. A linear calibration model with weighting $1/x^2$ was chosen. The correlation coefficient for SRM 1 and SRM 2 was 0.9980 ± 0.0016 (range: 0.9954–0.9996) for PEth 16:0/18:1. The correlation coefficient for SRM 1 and SRM 2 was 0.9974 ± 0.0005 (range: 0.9953–0.9982) for PEth 16:0/18:2. Extended calibration ranges were tested, with additional calibration samples at 1,750, 2,000, 2,250, 2,500 and 3,000 ng/mL. An extended linear range up to 2,500 ng/mL was investigated for all four monitored transitions with a correlation coefficient of at least 0.9975. The LOQ was set at 20 ng/mL, as samples below this concentration are associated with light or no alcohol consumption. Inaccuracy and imprecision at this concentration were below 15%. The LOD was observed at 10 ng/mL based on the repetitive analysis of samples at this concentration with an observed signal-to-noise ratio of at least 3.

Accuracy and precision

Accuracy and precision were investigated during the validation process by the analysis of spiked samples at the quality control concentrations. Six individual blood spots at each quality control concentration were analyzed on three different days, see Table II. Accuracy and precision remained within the required ±15% during intra-assay and inter-assay evaluation for all three measurements.

Selectivity

The chromatography has been optimized for both analogs, PEth 16:0/18:1 and PEth 16:0/18:2. Base peak separation successfully prevented cross-talk between PEth 16:0/18:2-2 × ^{13}C and PEth 16:0/18:1 (relative abundance of 8.7%). Samples from six alcohol abstinent subjects were examined for chromatographic interferences. There were no interfering peaks observed with the same retention time as PEth 16:0/18:1 or PEth 16:0/18:2 in all of the investigated samples.

Carry-over

Carry-over was tested in each run by the injection of a blank sample directly after the highest calibrator (K6, 1,500 ng/mL), and was found to be up to 0.5% (7.5 ng/mL).

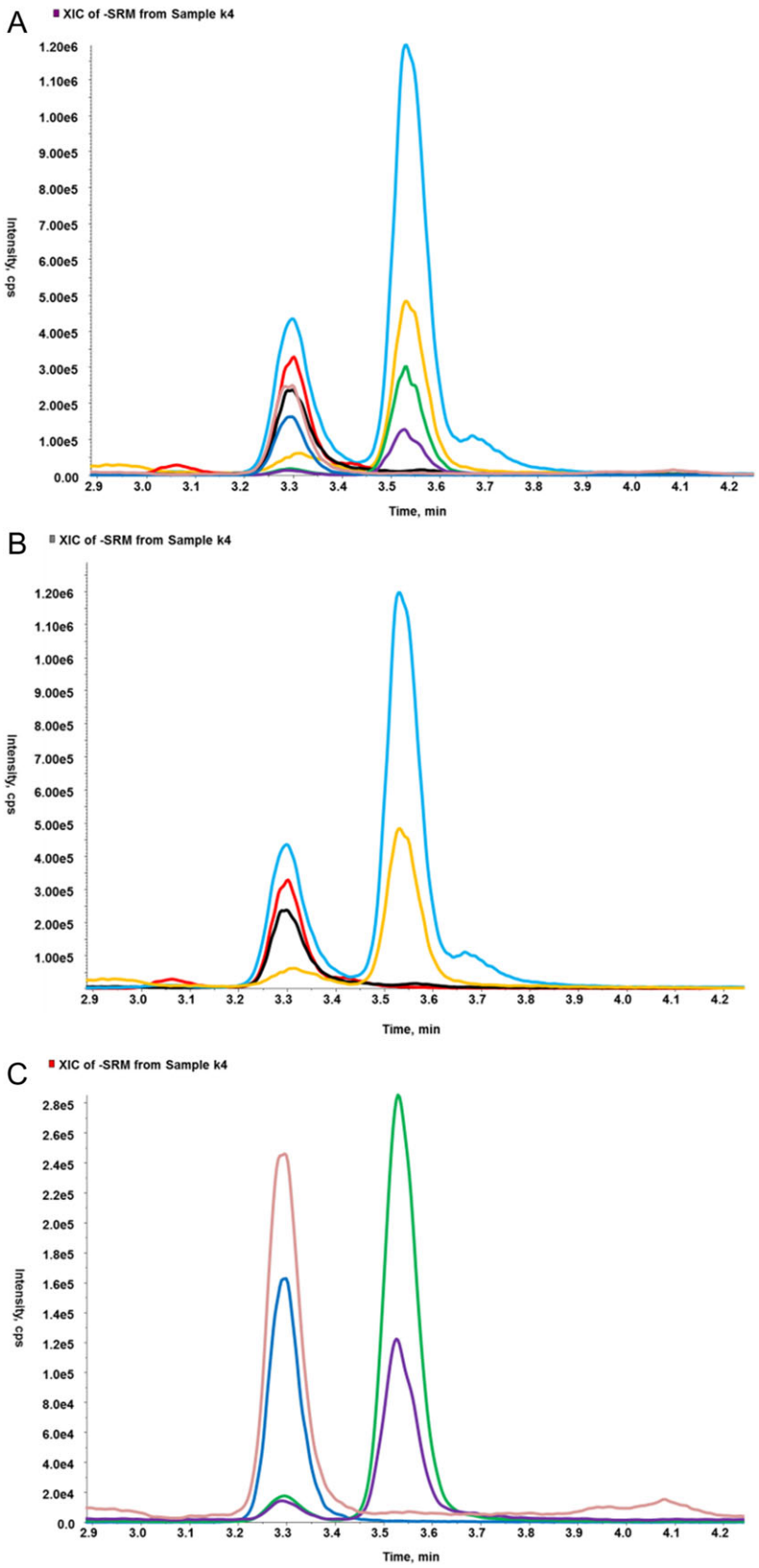

Figure 2. Chromatogram for a calibrator sample spiked at K4 (375 ng/mL). Part (A) represents all monitored SRM transitions. Part (B) represents the monitored analyte SRM transitions. Part (C) represents the monitored internal standard SRM transitions. Orange: PEth 16:0/18:1 SRM 1, red: PEth 16:0/18:2 SRM 1, cyan: PEth 16:0/18:1 SRM 2, black: PEth 16:0/18:2 SRM 2, green: PEth 16:0/18:1–D_5 SRM 1, blue: PEth 16:0/18:2–D_5 SRM 1, purple: PEth 16:0/18:1–D_5 SRM 2, wine: PEth 16:0/18:2–D_5 SRM 2.

Table II. Intra-assay and inter-assay accuracy and imprecision determined during three validation runs with six quality control samples at each concentration

Analyte	Concentration (ng/mL)	Intra-assay accuracy (%, $n = 6$)	Inter-assay accuracy (%, $n = 3$)	Intra-assay imprecision (%, $n = 6$)	Inter-assay imprecision (%, $n = 3$)
PEth 16:0/18:1 SRM 1	20	95–104	100.0	3.7–4.3	4.57
	45	102–106	103.3	2.7–4.6	1.99
	1,180	87–97	91.2	2.5–6	5.69
PEth 16:0/18:1 SRM 2	20	97–101	98.9	2.5–4.3	2.11
	45	97–99	98.5	2.8–6.6	1.16
	1,180	93–104	98.0	2.3–5.1	5.82
PEth 16:0/18:2 SRM 1	20	97–103	99.3	2.8–7.4	3.12
	45	94–105	98.3	3.3–6.7	5.84
	1,180	100–107	103.7	2.9–5.1	2.89
PEth 16:0/18:2 SRM 2	20	97–106	100.8	3.4–8.3	4.52
	45	97–100	99.2	3.2–4.9	1.91
	1,180	93–104	95.1	3.3–6.5	8.14

Matrix effects

Matrix effects were tested by the injection of matrix extracts from six different teetotallers spiked with PEth concentrations at K3 and K6. Each sample was prepared in duplicate. The internal standard corrected concentration was compared to samples containing no matrix, spiked at concentration K3 and K6. The obtained results are listed in the Supplementary Material S1. Thus, the investigated matrix effects can be considered as negligible when compensated with deuterated IS. Ion enhancement and ion suppression were investigated by comparing the analyte area without any internal standard correction: For PEth 16:0/18:1 a response of 97–129% and for PEth 16:0/18:2 a response of 123–198% was observed.

Extraction efficiency

Extraction efficiency was investigated by the repetitive extraction of calibrator samples. Thereby calibrator samples at K4–K6 (375–1,500 ng/mL) were extracted six times. The monitored analyte peak area after each extraction was thereby compared to the total analyte peak area obtained after six extractions. For PEth 16:0/18:1, a mean extraction efficiency of 89% after the first extraction, and 7% after the second extraction was observed. For PEth 16:0/18:2 a mean extraction efficiency of 88% after the first extraction, and 8% after the second extraction was observed.

Application of internal standard

To investigate if the time point of the internal standard application affects the outcome of the measurement, two different approaches were compared: on the one hand direct application of the internal standard before the extraction, on the other hand, application of the internal standard 8 h before the extraction.

Considering the quantification of 28 real case samples from alcohol use disorder (AUD) patients, no differences were observed: The mean agreement was 105 ± 10% ($R = 0.9821$) for PEth 16:0/18:1 SRM 1 and 101%±10% ($R = 0.9832$) for PEth 16:0/18:1 SRM 2. For PEth 16:0/18:2 SRM 1, a mean agreement of 93 ± 10% ($R = 0.9897$), and for PEth 16:0/18:2 SRM 2 of 95%±16% ($R = 0.9877$) was observed.

Comparison with PEth in liquid whole blood

To compare the fully automated extraction with the determination of PEth in liquid whole blood, 28 samples from AUD patients were compared.

A comparison between PEth in liquid whole blood and fully automated DBS extraction revealed that PEth results obtained with both methods are comparable: For PEth 16:0/18:1 SRM 1 a mean agreement of 100 ± 9% (range: 84–128%), and for PEth 16:0/18:1 SRM 2 a mean agreement of 101 ± 9% (range: 81–122%) was observed. For PEth 16:0/18:2 SRM 1 a mean agreement of 98 ± 8% (range: 87–118%), and for PEth 16:0/18:2 SRM 2 a mean agreement of 99 ± 9% (range: 82–116%) was observed. The linearity of the two methods of PEth determination was high, see Figure 3.

Comparison between manual and automated DBS extraction

To compare the fully automated extraction with manual DBS extraction, 28 DBS samples from AUD patients were compared.

A comparison between the manually extracted DBS and fully automated extraction revealed that PEth results obtained with both methods are comparable: For PEth 16:0/18:1 SRM 1 a mean agreement of 108 ± 11% (range: 87–125%), and for PEth 16:0/18:1 SRM 2 a mean agreement of 107 ± 10% (range: 87–126%) was observed. For PEth 16:0/18:2 SRM 1 a mean agreement of 96 ± 10% (range: 75–117%), and for PEth 16:0/18:2 SRM 2 a mean agreement of 95 ± 10% (range: 79–118%) was observed. The linearity of the two methods of PEth determination was high, see Figure 4.

Discussion

The aim of this study was the development of a method for the fully automated sample preparation, extraction and online LC-MS/MS quantification of PEth 16:0/18:1 and PEth 16:0/18:2. In contrast to only analyzing the most abundant PEth species, PEth 16:0/18:1, the method presented here has a distinct advantage: the quantification of the two most abundant PEth homologs allows confirmation of the result within the run. In abstinent subjects, both of the analogs may be present at very low concentrations (<20 ng/mL); in AUD patients, on the other hand, both analogs are present at elevated concentrations.

During the validation process, the automated extraction method proved to be a fast and reliable method for the determination of PEth samples. The method proved to be linear, accurate and precise. Considering the linearity, the observed correlation coefficient was at least 0.9953. Accuracy and precision remained below the required ±15% for all of the monitored transitions, independent of intra-

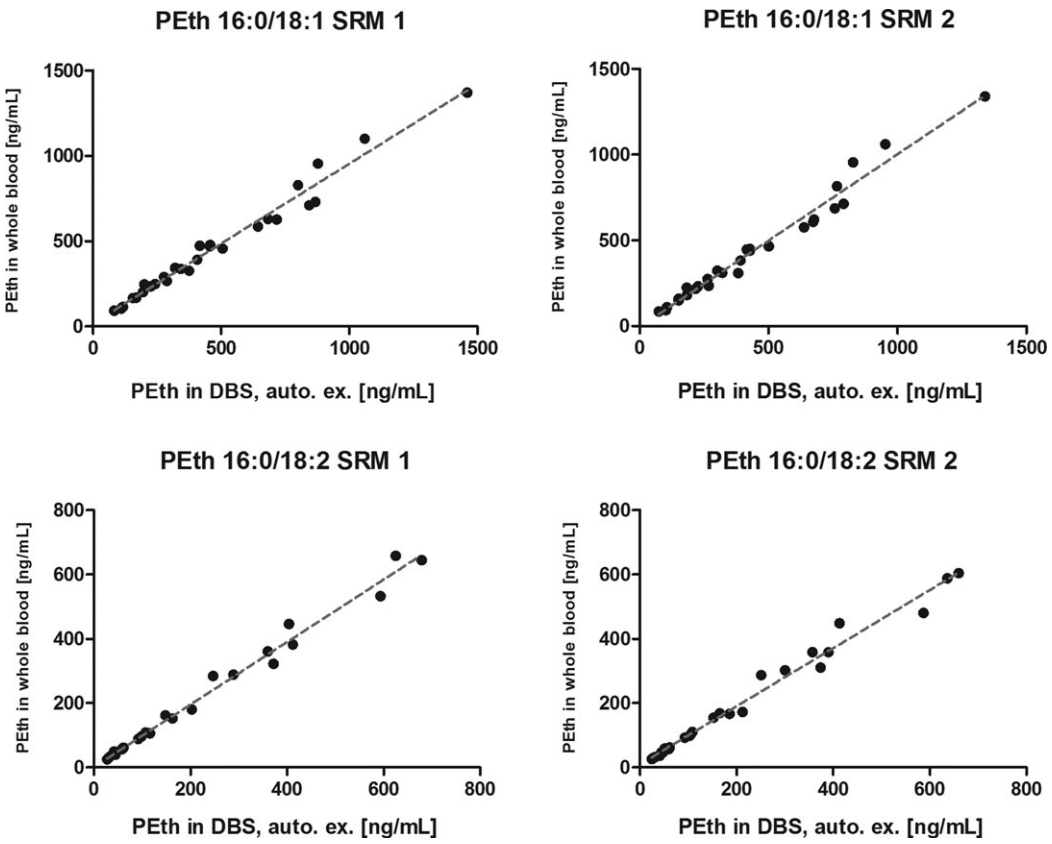

Figure 3. Comparison of PEth concentrations determined in liquid whole blood and automated DBS extraction for 28 samples from AUD patients (data points above LOQ). PEth 16:0/18:1 SRM 1: Slope: 0.9349 ± 0.02824 ($R = 0.9768$), $P < 0.0001$. PEth 16:0/18:1 SRM 2: Slope: 1.007 ± 0.03102 ($R = 0.9759$), $P < 0.0001$. PEth 16:0/18:2 SRM 1: Slope: 0.9706 ± 0.02339 ($R = 0.9863$), $P < 0.0001$. PEth 16:0/18:2 SRM 2: Slope: 0.9029 ± 0.02489 ($R = 0.9821$), $P < 0.0001$.

assay or inter-assay comparison. The LOQ of 20 ng/mL permits the identification of samples at common PEth reference interval concentrations such as 35 ng/mL (19).

Furthermore, no interferences were observed for blood samples from teetotallers. By the use of two internal standards for PEth 16:0/18:1 and 16:0/18:2 it was possible to compensate for potential matrix effects occurring throughout the measurement. In addition, the application of internal standard directly to the DBS by the spray module allows compensating for potential extraction bias. In comparison to manual DBS extraction using passive elution from the spot using shaking in solvent, the active extraction under pressure showed high extraction efficiencies of about 90%. In contrast, Kummer *et al.* reported PEth extraction efficiencies for manual DBS extraction >55% (RSD < 18%) (20).

Concerning the investigated carry over of up to 0.5%, we recommend the reanalysis of samples close to the lower limit of reporting, if preceded by a sample with a high concentration. Another option is the extraction of a blank after each real case sample. For the manual injection of liquid extracts, comparable carry over was investigated, leading to the conclusion, that the carry over may be related to the column material. In general, PEth appears to have properties which lead to minimal carry over: Isaksson *et al.* reported carry over of about 0.2% for their high-throughput method for whole blood (19).

As the sample preparation is performed within the autosampler unit, laboratory work is reduced to an absolute minimum: only the calibrator and quality control samples have to be prepared manually. However, this can be performed in advance of the analysis, as PEth was found to be relatively stable on DBS (21). The potential of the automated system for high-throughput analysis of PEth provides a short turnaround time for large quantities of samples. Based on low-key calculation with a run time of about 8 min, 180 samples can be analyzed each day. This simplified and fast acquisition method may lead to a broader acceptance of PEth for routine analysis.

By comparing samples from AUD patients which contain endogenous PEth, it could be shown that the PEth concentrations determined by the fully automated system are comparable to the measurement in liquid whole blood or manual DBS extraction. This allows results to be comparable independent of the applied extraction method. To be comparable, the protocol for the manual calibration and quality control sample preparation had to be prolonged, as differences concerning the results between authentic PEth samples from AUD patients were observed between the manual PEth DBS extraction and the automated PEth DBS extraction. The manually prepared quality control samples with spiked PEth did not require the prolonged incubation protocol, as no differences between the extraction methods were observed for these samples. This implies that there is a difference between PEth that is integrated into the blood cells and exogenous PEth which is spiked into blood from a teetotaller. However, prolonged incubation of spiked PEth and blank blood appears to successfully incorporate PEth, leading to comparable results. Therefore, careful cross-validation between different extraction methods with authentic PEth samples is highly recommended for the validation of PEth methods.

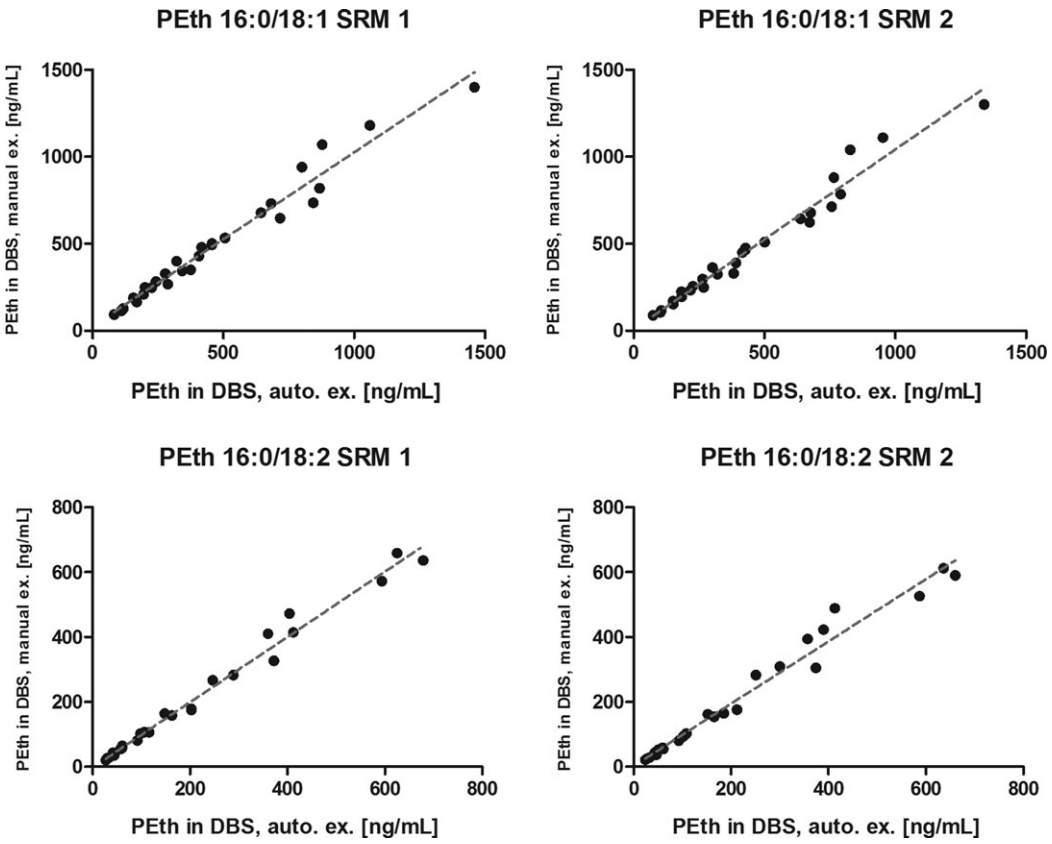

Figure 4. Comparison of manual DBS extraction and automated DBS extraction for 28 samples from AUD patients (data points above LOQ). PEth 16:0/18:1 SRM 1: Slope: 0.9995 ± 0.03644 ($R = 0.9666$), $P < 0.0001$. PEth 16:0/18:1 SRM 2: Slope: 1.033 ± 0.03613 ($R = 0.9692$), $P < 0.0001$. PEth 16:0/18:2 SRM 1: Slope: 1.006 ± 0.02542 ($R = 0.9849$), $P < 0.0001$. PEth 16:0/18:2 SRM 2: Slope: 0.9602 ± 0.03170 ($R = 0.9745$), $P < 0.0001$.

Conclusion

A new workflow for high-throughput determination of PEth in DBS was developed by automated sample work-up with a DBS-MS 500 coupled to online-SPE-LC-MS/MS.

The analytical process is fully automated by using an online DBS-SPE-LC-MS/MS analysis system. The feasibility of the automated analysis was successfully validated and has been applied to real case samples. The obtained results are comparable to the widely applied manual extraction of PEth in DBS or the analysis of PEth in liquid blood samples. Automation with the DBMS-500 includes the possibility of sample identification by a barcode label on the DBS card—with a link to a laboratory information system (LIMS).

Supplementary data

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

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