High throughput UPLC®-MSMS method for the analysis of phosphatidylethanol (PEth) 16:0/18:1, a specific biomarker for alcohol consumption, in whole blood

Trine Naalsund Andreassen[a,*], Hilde Havnen[a], Olav Spigset[a,b], Berit Margrethe Hasle Falch[a], Ragnhild Bergene Skråstad[a,b]

[a] Department of Clinical Pharmacology, St. Olav University Hospital, Trondheim, Norway

[b] Department of Laboratory Medicine, Children's and Women's Health, Norwegian University of Science and Technology, Trondheim, Norway

*Corresponding author at: Department of Clinical Pharmacology, St. Olav University Hospital, 7006 Trondheim, Norway. Phone: +47 72 82 91 39. E-mail address: trine.naalsund.andreassen@stolav.no

Abstract

Phosphatidylethanol (PEth) is an alcohol biomarker formed in the presence of ethanol in the body. Both 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. The aim of this study was to develop and validate a robust method for PEth in whole blood with fast and efficient sample extraction and a short analytical runtime, suitable for high throughput routine purposes. A validated ultra-performance liquid chromatography tandem mass spectrometry (UPLC®-MSMS) method for quantification of PEth 16:0/18:1 in the range 0.05-4.00 µM ($R^2 \geq 0.999$) is presented. PEth 16:0/18:1 and the internal standard PEth-d_5 (0.55 µM), were extracted from whole blood (150 µl) by simple protein precipitation with 2-propanol (450 µl). Chromatography was achieved using a BEH-phenyl (2.1 x 30 mm, 1.7 µm) column and a gradient elution combining ammonium formate (5 mM, pH 10.1) and acetonitrile at a flow rate of 0.5 ml/min. Runtime was 2.3 minutes. The mass spectrometer was monitored in negative mode with multiple reaction monitoring (MRM). The m/z 701.7 > 255.2 and 701.7 > 281.3 transitions were monitored for PEth 16:0/18:1 and the m/z 706.7 > 255.3 for PEth-d_5. Limit of quantification was 0.03 µM (CV = 6.7 %, accuracy = 99.3 %). Within-assay and between-assay imprecision were 0.4 – 3.3 % (CV ≤ 7.1 %). Recoveries were 95-102 % (CV ≤ 4.9 %). Matrix effects after internal standard correction ranged from 107-112 %. PEth 16:0/18:1 in patient samples were stable for several days at 30 °C. Repeated freezing (-80 °C) and thawing did not affect the concentration. After thawing and analysis patient samples were stable at 4-8 °C for at least four weeks. Results from a proficiency test program, showing |Z| values ≤ 1.2, confirm the validity of the method. Analysis of the first 3169 samples sent to our laboratory for routine use has demonstrated its properties as a robust method suitable for high throughput purposes.

Keywords

PEth 16:0/18:1, alcohol biomarker, UPLC®-MSMS, quantification, protein precipitation, stability

Introduction

High alcohol consumption is a risk factor for developing a range of mental as well as somatic diseases, to be involved in accidents and to commit and be subjected to criminal acts. Harmful consumption of alcohol is a major health issue both in industrialized and non-industrialized parts of the world. The World Health Organization estimates that 3.3 million deaths annually (i.e. 5.9 % of all deaths) are caused by alcohol consumption (1). It is therefore a key task for health care systems to contribute to the reduction of harmful alcohol use. As subjects with potentially harmful patterns of alcohol consumption tend to underestimate their alcohol intake, objective measures of alcohol consumption are crucial in the diagnosis as well as the follow up of alcohol-related conditions. There is therefore a great need for a sensitive and specific alcohol biomarker that can be analyzed in an effective and accurate manner.

Phosphatidylethanol (PEth) is a collective term for abnormal phospholipids formed from fatty acids by the enzyme phospholipase D in the membranes of red blood cells in the presence of ethanol (2, 3). The molecular structure of PEth consists of a phosphoethanol head group on which two fatty acid chains are attached. The length of the fatty acid chains can vary from 14 to 22 carbons and these may have up to six unsaturated bonds. So far, 48 homologues of PEth have been identified (4) and PEth 16:0/18:1 and PEth 16:0/18:2 are the most abundant (4-8).

PEth can be used as a direct biomarker of alcohol consumption. It has a half-life of 3-5 days (9, 10), possibly longer in persons with low to moderate alcohol consumption and towards the end of the elimination phase (11), and a detection window of up to three (12) to four weeks has been reported (10). The formation of PEth is specific for ethanol (13), and age, gender and liver disease do not seem to affect the relationship between alcohol intake and PEth levels (14). In vitro studies indicate a linear relationship between alcohol concentrations in blood, exposure time and PEth formation (15). Therefore, the application potential for this alcohol biomarker is expected to be broad (11, 16-20). In contrast to traditional indirect biomarkers like mean cellular volume (MCV), gamma glutamyl transpeptidase (GGT) and carbohydrate deficient transferrin (CDT %), which can be affected by age, gender and non-alcohol-associated diseases, PEth is specific for alcohol intake. It has also been demonstrated that PEth surpasses MCV and GGT and possibly also CDT % both for the identification of a high/excessive alcohol intake (10, 17) as well as for the differentiation between sobriety and any alcohol intake (11, 16, 19).

For the last years LC-MSMS has been the major analytical method of choice for quantification of PEth due to the sensitivity needed to detect low alcohol consumption (6, 11, 21-25). In these applications liquid-liquid extraction involving hexane, heptane or dichloromethane, solely or in combination with 2-propanol, have been used for the extraction of PEth followed by time-consuming evaporation and reconstitution. Runtimes have varied from 3.5 to 22 minutes (11, 21, 23, 25-27).

The PEth applications reporting the lowest limit of quantifications (LOQs) all involve manual sample extraction using potentially toxic extraction solvents and the time-consuming evaporation-reconstitution step (28-30). Only one of these low LOQ studies revealed the analytical runtime, which in that case was 10 minutes (30). Summarized, these applications are more suited for clinical research, involving few samples than for routine use. Due to the large volume of samples submitted to our laboratory, we aimed to develop a robust method with a fast and efficient sample extraction and a short analytical runtime. We here present our high throughput ultra-performance liquid chromatography tandem mass spectrometry (UPLC®-MSMS) method for quantification of PEth 16:0/18:1 in whole blood for clinical routine use.

Experimental

Chemicals and reagents

1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanol (PEth 16:0/18:1) 1 mM in chloroform was purchased from Chiron (Trondheim, Norway) and 1-palmioyl-2-oleoyl-sn-glycero-3-phospho(ethanol-d_5) (PEth-d_5) 10 mg/ml in chloroform was purchased from Avanti Polar lipids (InstruChemie, Delfzijl, The Netherlands). Ammonium formate (99.9 %) and ammonium hydroxide solution (ACS-reagens 28.0 -30.0 % NH_3-basis) were purchased from Sigma Aldrich (Oslo, Norway). 2-propanol of LC-MS grade and acetonitrile Hyper LC-MS grade were purchased from various commercial sources. Human whole blood was obtained from blood donors abstaining from alcohol use (St. Olav University Hospital, Trondheim, Norway).

Stock solutions of phosphatidylethanol were prepared to a concentration of 1 mM in 2-propanol and these were further diluted with 2-propanol to yield working solutions for calibrators and quality control (QC) samples. The working solutions were used to spike calibrators and QCs in whole blood. Stock solutions and working solutions were stored at -20 °C. The internal standard PEth-d_5 was diluted in 2-propanol to a concentration of 0.55 µM and stored at 4-8 °C.

Sample preparation

Prior to analysis, the patient samples were stored at – 80 °C to induce hemolysis. This was done to prevent sedimentation of the patient EDTA-samples during the time of the automatic extraction. This storage temperature also prevents *in vitro* formation of PEth after sampling in case of ethanol being present in the blood at the time of sample collection (8, 31).

After thawing to room temperature, automatic sample preparation was performed using a Tecan Freedom Evo pipetting robot (Tecan, Männedorf, Switzerland). PEth-d_5 (0.55 µM, 350 µl) in 2-propanol was pipetted into a 2 ml collection plate (96-well Square collection plate, Waters corp., Milford, MA, USA). After addition of 2-propanol (100 µl), aliquots of patient samples (150 µl) were added. Calibrators and QCs were prepared by adding working solution (100 µl) and whole blood (150 µl) onto the collection plate containing the PEth-d_5 solution. The collection plate was sealed with tinfoil (Miniseal 2 Automatic single Plate Heat sealer, Porvair, Teknolab, Oslo, Norway), mixed at 2100 rpm for 1 minute, (Co-mix Multi-format Plates & Tubes, Teknolab, Oslo, Norway) and centrifuged at 4600 rpm for 5 minutes (Rotana 460, Hettich Zentrifugen, Tuttlingen, Germany).

UPLC®-MSMS

All experiments were carried out on a UPLC® system (Waters Acquity System, Waters, Manchester, UK) coupled to a tandem-quadrupole mass spectrometer (Xevo TQS, Waters, Milford, MA, USA).

UPLC® conditions

Chromatography was performed using a Waters Acquity UPLC® BEH-Phenyl column (2.1 x 30 mm, 1.7 µm) with a Waters Van Guard™ BEH-Phenyl pre-column (2.1 x 5 mm, 1.7 µm) and oven temperature 50 °C. The gradient elution was performed with ammonium formate (5 mM, pH 10.1, mobile phase A) and acetonitrile (mobile phase B) at a flow rate of 0.5 ml/min. The gradient run was from 60 % to 95 % of B during the first 90 seconds and then kept with 95 % of B for 6 seconds. 42 seconds with 60 % B was found sufficient to equilibrate the column before next injection. Thus, the total runtime was 138 seconds, i.e. 2 minutes and 18 seconds. The injection volume was 2.0 µL and the autosampler temperature was set to 10 °C.

Mass spectrometry

The MS/MS-detection was performed with electrospray ionization (ESI) in the negative mode and multiple reaction monitoring (MRM). The capillary voltage was set to -2.0 kV, the source block temperature was 120 °C and the nitrogen desolvation gas was heated to 650 °C with a flow rate of 1000 L/h. The m/z 701.7 > 255.2 and 701.7 > 281.3 transitions (cone voltages 94 V, collision energy 38 and 30 eV, respectively) were monitored for PEth 16:0/18:1 and the m/z 706.7 > 255.3 transition (cone voltages 96 V, collision energy 38) was monitored for PEth-d_5. Cross-talk between PEth 16:0/18:1 and the internal standard PEth-d_5 was first checked by monitoring the internal standard MRM transitions m/z 706.7 > 281.3 and m/z 706.7 > 255.3 after injection of a PEth 16:0/18:1 (4.00 µM) sample in 2-propanol. Secondly, a 2-propanol solution only spiked with the internal standard, to a concentration level equivalent to an authentic sample being analyzed, was analyzed and the PEth 16:0/18:1 MRM transitions m/z 701.7 > 281.3 and 701.7 > 255.2 were checked.

System operation and data acquisition were controlled using Mass Lynx 4.1 software (Waters). All data were processed with the Target Lynx quantification program (Waters). PEth 16:0/18:1 was identified by comparing the retention time and the mass spectra quantifier-qualifier ratio of the corresponding calibrator and QC samples.

Method validation

The validation process included selectivity, calibration model, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy, extraction recovery, matrix effects, carryover, ion ratio and stability.

Selectivity

Whole blood from six blood donors was analyzed and checked for interference by comparing whole blood chromatograms to chromatograms with PEth 16:0/18:1 concentrations corresponding to the LOQ. Interference should not exceed 15 % of the LOQ sample area.

Calibration curves

Calibrators were prepared by spiking whole blood at 0.05, 0.10, 1.00, 2.00 and 4.00 µM. QCs were prepared by spiking whole blood at 0.08 µM (QC1), 0.50 µM (QC2) and 3.00 µM (QC3). The calibration curve was assessed with three replicates of each level of the calibrators. The calibration was performed by peak-area ratios of the analyte relative to the internal standard using weighted (1/x) quadratic regression, excluding the origin.

Limit of detection (LOD) and limit of quantification (LOQ)

LOD was determined by evaluation of signal-to-noise (S/N) for extracted dilutions of a low calibrator with acceptance criteria for S/N > 3 for both transitions.

LOQ was determined by spiking whole blood with various concentrations in the range of 0.01 – 0.05 µM. Samples of 0.02 and 0.03 µM were run in one replicate on ten different days, and the concentration was calculated using a calibration curve ranging from 0.02 to 1.00 µM. The S/N criteria for the LOQ samples were ≥ 10 and the precision acceptance criteria for the calculated concentrations were within ± 20 %.

Precision and accuracy

Within-assay precision was estimated by analysis of six replicates of QC samples at the three concentration levels (QC1, QC2 and QC3) in a single assay. Acceptance criteria for within-assay precision were a coefficient of variation (CV) < 10 %.

Between-assay precision was determined by analysis of one replicate at the three QC concentrations on ten different days. Concentrations were calculated from daily calibration curves. Between-day precision acceptance criteria were CV ≤ 20 %.

The between-assay precision data were used to calculate the method accuracy with an acceptance criterion of deviation ≤ 15 %. Also, analyses of six external quality control samples (Equalis, Uppsala, Sweden) were included. Z scores were calculated as the difference between our result and the consensus mean or median of the results of the participating laboratories and divided by the combined SD of the inter-laboratory variation for our method. The acceptance criteria when analyzing the external quality control samples was |Z| values ≤ 2.

Extraction recovery and matrix effects

Extraction recoveries (ERs) were determined at low and high (QC1 and QC3) concentration levels with six replicates for each level. ER was calculated by dividing analyte/internal standard (IS) area ratio of blood spiked and protein precipitated (A) by analyte/IS area ratio of blood spiked after protein precipitation (B); ER = (A/B) × 100 %.

Matrix effects (MEs) were evaluated at two concentration levels (QC1 and QC3) with post-extraction spiked blood from six different donors according to the method proposed by Matuszewski et al. (32) . The post-extraction samples (C) were compared to a 2-propanol solution spiked with PEth 16:0/18:1 (D) to a concentration equivalent to the concentration of the post-extraction samples. Matrix effects were calculated as ME (%) = $(C_{area})/(D_{area})$ × 100 %, and, when corrected with the IS, ME (%) = $(C_{area}/IS_{area})/(D_{area}/IS_{area})$ × 100 %. Acceptance criteria for matrix effects were 75-125 % with CVs ≤ 15 %.

Carryover

Carryover on the UPLC® system was assessed by comparing the chromatography of the lowest calibrator with the chromatography of a blank blood sample analyzed after injection of a spiked 8.00 µM PEth 16:0/18:1 sample. The acceptance criterion for carryover was < 20 % of the lowest calibrator (0.05 µM).

Quantifier-qualifier ion ratio

Ion ratio stability of the 255.2 quantifier ion and the 281.3 qualifier ion was monitored on three days by running series of calibrators, QC and patient samples with a total of 90 injection. The acceptance criterion for ion ratio was deviation ≤ 20 % when compared to the reference ratio (mean calculated ratio of calibrator 2 and 3 in the same sequence run).

Stability

Stability in patient samples was tested at various temperatures and time intervals. Post-extraction stability was tested for 5 days at 10 °C. When the decrease in mean concentration was less than 15 % and the maximum decrease in any sample was less than 20 %, samples were defined to be stable under that particular condition.

Patient samples at 30 °C

Non-frozen patient samples (n = 7) in EDTA tubes were analyzed on the day of arrival at the laboratory and then stored at 30 °C in darkness before re-analysis after three and five days.

Thawed patient samples at 4-8 °C

Patient samples were stored at -80 °C before analysis. Patient samples (n = 6) were thawed on the day of analysis (Day 0) and then stored at 4-8 °C before re-analysis after one, two, three and four weeks.

Freeze-thaw stability

Patient samples (n = 6) were thawed from -80 °C to room temperature and aliquots (0.5 ml) from each sample were stored in polystyrene tubes at 4-8 °C while the original patient EDTA tubes were frozen and thawed three times within four hours. All samples were allowed to reach room temperature before sample preparation and analysis.

Post extraction stability

Post-extraction stability was evaluated by analyzing a set of calibrators and one replicate of QC1, QC2 and QC3 together with patient samples (n = 7) on the extraction day. After being stored at 10 °C in the autosampler in darkness for three and five days the extracted samples were re-injected and analyzed.

Results and discussion

A method for PEth quantification with 2-propanol protein precipitation and direct injection is presented. A Tecan pipetting robot was used for automatic sample extraction, but this can also be done manually if desired. The total ion and multiple reaction monitoring chromatograms for PEth 16:0/18:1 are shown in Figure 1.

As part of the validation, transport and storage conditions were also tested as appropriate handling and storage of the patient samples is a prerequisite for a reliable analytical answer.

48 homologues of PEth have previously been identified (4). Of these homologue 16:0/18:2 with MRM transitions m/z 699.5 > 255.2 (16:0) and m/z 699.5 > 279.2 (18:2), homologue 16:1/18:0 with MRM transitions m/z 701.5 > 253.2 (16:1) and m/z 701.5 > 283.2 (18:0) and homologue 16:1/18:1 with MRM transition m/z 699.5 > 253.2 (16:1) and m/z 699.5 > 281.2 (18:1) have molecular ions that are very close in total mass or fragment ion mass to PEth 16:0/18:1. The sample extraction will not discriminate between any of the PEth homologues, but by combining chromatography for separation of the homologues together with a triple quadrupole mass spectrometer in MRM mode, it is possible to measure the homologue of interest. For quantification it is required that both the

quantifier and the qualifier ion exist, and the ratio must pass the acceptance criterion. Chromatography in combination with two MRM transitions makes up the selectivity of the analytical method.

As shown in Figure 2, there is no cross-talk between PEth 16:0/18:1 and the internal standard for the MRM transitions chosen in this method.

Method validation

The validation results are summarized in Table 1.

Selectivity

Two of the six whole blood samples tested had interfering peaks. The areas of these interfering peaks accounted for more than 15 % of a spiked 0.02 µM sample and less than 1.5 % of a spiked 0.03 µM sample. Standard 0 (whole blood with IS added) was analyzed for ten different days without showing any interference.

Calibration curves

In the range of 0.05-4.00 µM the method showed a squared coefficient of determination (R^2) ≥ 0.999. The weighted (1/x) residuals were found to spread randomly around zero (Figure 3). R^2 ≥ 0.999 was also obtained when the method was tested for the 0.05-8.00 µM or 0.03-4.00 µM range.

Limit of detection (LOD) and limit of quantification (LOQ)

LOD was set to 0.01 µM with an S/N value of 26 for the 701.7 > 255.2 transition and 10 for the 701.7 > 281.3 transition. LOQ was set to 0.03 µM (CV = 6.7 %, accuracy = 99.3 %) with S/N ≥ 16 for both transitions.

Precision and accuracy

The CVs for within-assay precision were ≤ 5.1 %, 1.1 % and 1.1 % for the QC1, QC2 and QC3 levels, respectively. For the between-assay precision the CVs were ≤ 7.1 %, 4.1 % and 2.2 % with inaccuracies of 3.3 %, 2.4 % and 0.4 % for the QC1, QC2 and QC3 levels, respectively. The external control samples (n = 6), with target values ranging from 0.16-2.5 µM, had all |Z| values ≤ 1.1. All results were in accordance with the acceptance criteria.

Extraction recovery and matrix effects

Extraction recoveries of 93-103 % (CV ≤ 5.1 %) without and 95-102 % (CV ≤ 4.9 %) with IS correction were obtained during the method validation.
Matrix effects ranged from 426-538 % (CV ≤ 11.8 %) without and from 107-112 % (CV ≤ 1.8 %) with IS correction. These results indicate a considerable effect from the matrix on the ionization of PEth 16:0/18:1; however, the deuterated IS corrects for this effect. This finding thus stresses the importance of using a deuterated, or other stable isotope labeled, internal standard for accurate measurements. PEth-d_5 was chosen as internal standard for this assay but PEth-d_{31}, which was tested during method development, seemed to work as well.

Carryover

The acceptance criterion for carryover was met. After injection of a sample with concentration twice the highest calibrator, carryover was 2.6 % of the lowest calibrator, only. Thus false positive results caused by carryover are not a problem.

Quantifier-qualifier ion ratio

After three days of monitoring and a total of ninety injections the highest deviation from the reference ratio measured was 10.2 %. The results met the acceptance criterion and demonstrate that the quantifier-qualifier ion ratio is stable for the method.

Stability

The main results from the stability tests are shown in Table 2.

Patient samples at 30 °C

Stability during transport to the analytical laboratory was assessed by exposure of patient samples (n = 7) to 30 °C and darkness. After three and five days, the concentration in the sample with the lowest PEth 16/1:18:0 level (0.07 µM) had dropped with 18.6 % and 17.1 %, respectively, compared to day 0. However, with this exception, the deviations from day 0 were very small. Thus, in general these results indicate good stability during these test conditions (Table 2).

Thawed patient samples at 4-8 °C

Stability of patient samples (n = 6) with concentrations ranging from 0.06 to 1.50 µM stored at 4-8 °C after analysis was tested for four weeks. The results show a concentration drop of ≤ 9.1 % after the test period, indicating good stability of the patient samples when stored up to four weeks (Table 2).

Freeze-thaw stability

There were no signs of degradation in patient samples (n = 6) after three freeze (-80 °C) and thaw cycles (Table 2). Thus, freezing and thawing do not seem to affect the concentration to any significant degree.

Post-extraction stability

There was no sign of degradation of PEth 16:0/18:1 dissolved in 2-propanol when kept in the autosampler at 10 °C for five days (Table 2). However, during the method development precipitations were observed in extracted samples stored at 4 °C. Thus, an autosampler temperature of 10 °C is recommended.

Experience from routine use

The presented method has been in routine use for more than a year and runs on a daily basis. As a crude extract of phospholipids is being injected on to the UPLC®-MSMS system, this can potentially contaminate and affect the performance of the instrument over time. We have during this period not detected any particular problems with the instruments or the method.

As a part of our routine all UPLC®-MSMS instruments go through a periodic maintenance once a year and sample cone and cone gas nozzle are regularly changed once a week. Apart from this, there has been no extra maintenance for these instruments compared to equivalent instrumentation used for other chromatographic drug of abuse or therapeutic drug monitoring methods.

The pre-column and column used for the analysis have shown to last for 3400-5000 injections. Our experience is that when the chromatography is poor both the pre-column and the main column have to be changed. Due to the nature of the extract, the retention time on the column is shifted approximately +6 seconds for the first injections on a new column. Then the retention time is gradually reduced during the first couple of hundreds injections before it stabilizes. However, this is not a problem as a deuterated internal standard is being used during the analysis. To prevent build-up

of phospholipids over time we routinely end every sequence run with 10 minutes of cleaning of the column with 100 % acetonitrile.

As a general precaution we always monitor carryover by analyzing a blank sample (blood with IS added) after the highest calibrator. So far, carryover on the system has not been observed.

It has been demonstrated that PEth can be formed *in vitro* if ethanol is present in the sample (15, 33); hence there is a risk of incorrectly high PEth values in samples drawn from inebriated patients. One way to handle this pitfall is to analyze all samples, or at least all samples positive for PEth, for ethanol, and discard PEth results from all ethanol positive samples. We have chosen not to undertake this procedure, as it would lead to increased use of resources in the laboratory and prolonged turnaround time for the samples. Instead, we inform the clinicians that sampling should be postponed if it is suspected that the patient is under influence of alcohol.

A total of 3169 consecutive and unselected analyses sent to our laboratory for routine purposes, were included in the study. According to the Regional Committee of Research Ethics, no formal approval is needed for a brief presentation of routine results a part of a methodological article. Of the samples included, 883 (27.9 %) were negative and 2286 (72.1 %) were positive using a cut-off of 0.05 µM. The distribution of the positive samples is displayed in Figure 4. Notably, only a very small proportion (n = 17; 0.5 %) had concentrations above 4.0 µM, which was the highest calibrator included in the method. The validity of the method is monitored on a regular basis by attendance of the external proficiency test program for PEth 16:0/18:1 organized by Equalis. For the 15 samples analyzed to date, with target values from 0.00-2.85 µM; Z-values have ranged from -1.2 to +0.5.

Interpretation of results and cut-off values

There is no international consensus on how the PEth 16:0/18:1 results should be reported or interpreted. Due to inter-individual variation in formation of PEth 16:0/18:1 (34) and the possibility of uncertain reporting on alcohol consumption in epidemiological studies, it is difficult to determine the amount of alcohol intake a given PEth 16:0/18:1 value represents (35). In Sweden a national harmonization was suggested in 2013, with concentrations above 0.3 µM indicating over-consumption of alcohol. Concentrations between 0.05 and 0.3 µM were suggested to represent moderate alcohol consumption, whereas concentrations below 0.05 µM were suggested to represent abstinence or an insignificantly low alcohol consumption (36).

However, recent studies have indicated that employing a cut-off of 0.05 µM might lead to a negative test result even though a certain amount of alcohol has been ingested (9, 11, 37). In an attempt to quantify this issue, we re-analyzed the 883 negative routine samples. We then found that 89 of these (i.e. 10 %) had a concentration above our LOQ of 0.03 µM, but below the lowest standard / cut-off value of 0.05 µM. Thus, the interpretation of a PEth 16:0/18:1 test result remains a challenge, and further studies regarding the relationship between alcohol consumption and PEth 16:0/18:1 formation and elimination are clearly needed.

Conclusion

We have developed and validated a high throughput UPLC®-MSMS method for quantification of PEth 16:0/18:1 in the range of 0.05-4.00 µM, with emphasis on fast and simple sample preparation and short analytical runtime. A robot is performing the extraction from the blood by protein precipitation with 2-propanol. The fully automated extraction simplifies the handling when many

samples are run in parallel; however, sample preparation can also easily be done manually. The calibration range can be extended downwards to 0.03 µM or up to 8.00 µM without compromising the linearity of the curve. Extraction recoveries were high and matrix effects were sufficiently corrected for by the internal standard. Extracted samples were stable for at least five days at the recommended storage temperature of 10 °C. PEth 16:0/18:1 in patient samples showed good stability at various temperatures and pre-analytical conditions up to 30 °C were tolerated for several days.

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Table 1 Summary of validation results

Calibration range	0.05-4.00 µM
Coefficient of variation, R^2	≥ 0.999
Limit of detection	0.01 µM
Limit of quantification	0.03 µM
Within-assay precision, CV	≤ 5.1 %
Between-assay precision, CV	≤ 7.1 %
Inaccuracy	≤ 3.3 %
Recovery*	95-102 %
Matrix effects*	107-112 %

*IS corrected

Table 2 Stability of patient samples after exposure to 30 °C, 4-8 °C, freeze-thaw cycles and post-extraction

Condition	Time	Mean deviation (%) from Day 0	Min dev. (%)	Max dev. (%)
30 °C and darkness	3 days	-9.6	-4.7	-18.6
	5 days	-9.2	-2.3	-17.1
4-8 °C and darkness	1 week	-0.6	0.2	-1.6
	4 weeks	-7.0	-3.7	-9.1
Freezing-thawing	3 cycles	-2.3	-0.6	-9.9
Post-extraction, 10 °C	3 days	0.5	0.8	-6.2
	5 days	-3.1	-0.7	-6.2

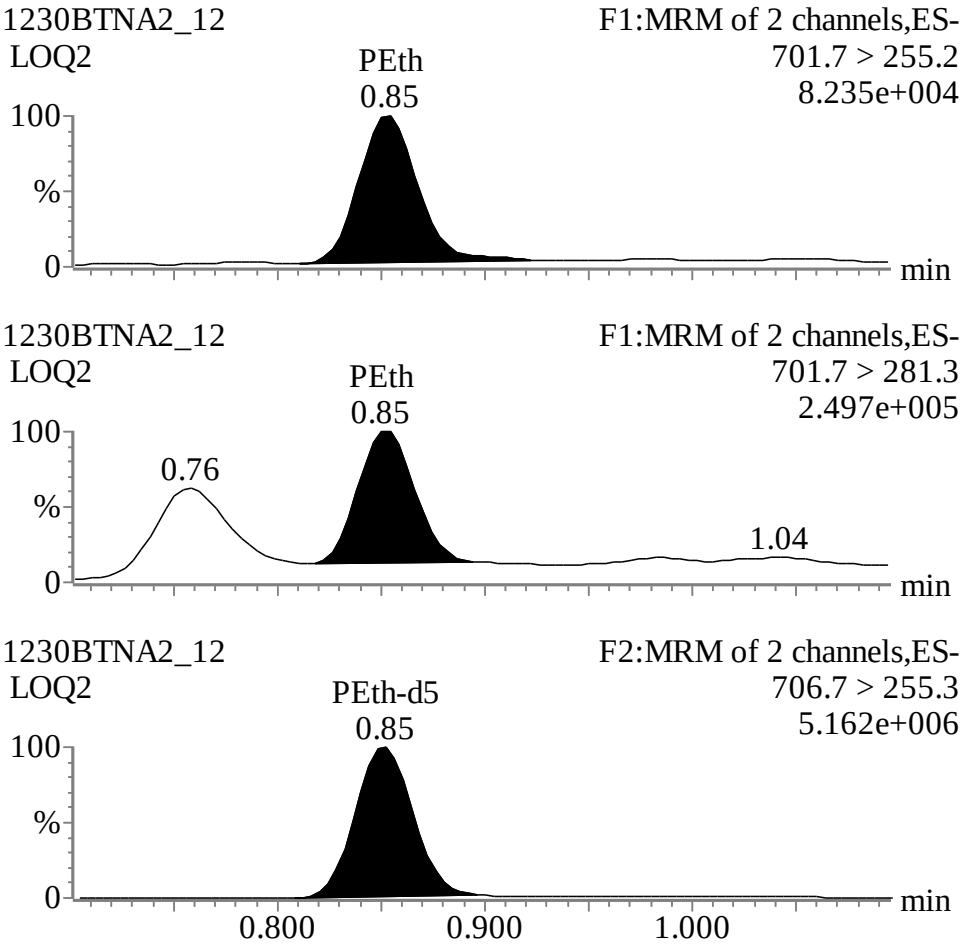

Figure 1 Total ion chromatogram (TIC) and multiple reaction monitoring (MRM) chromatograms for PEth 16:0/18:1 at a concentration equaling limit of quantification for the method, i.e. 0.03 µM.

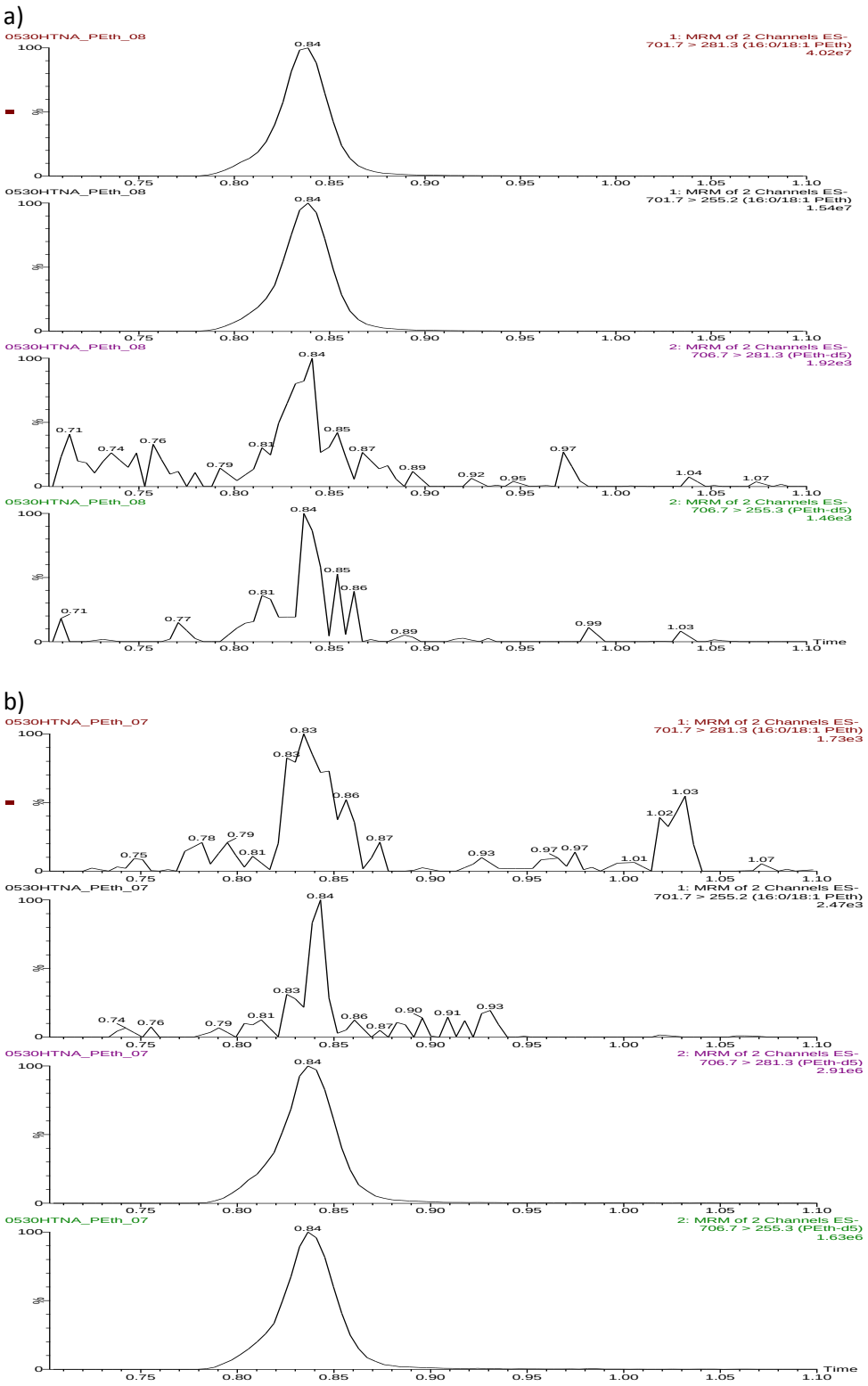

Figure 2 Absence of cross-talk for the multiple reaction monitoring (MRM) transitions used in the method; a) solution of 2-propanol spiked with 4.00 µM PEth 16:0/18:1 and b) solution of 2-propanol spiked with internal standard (IS).

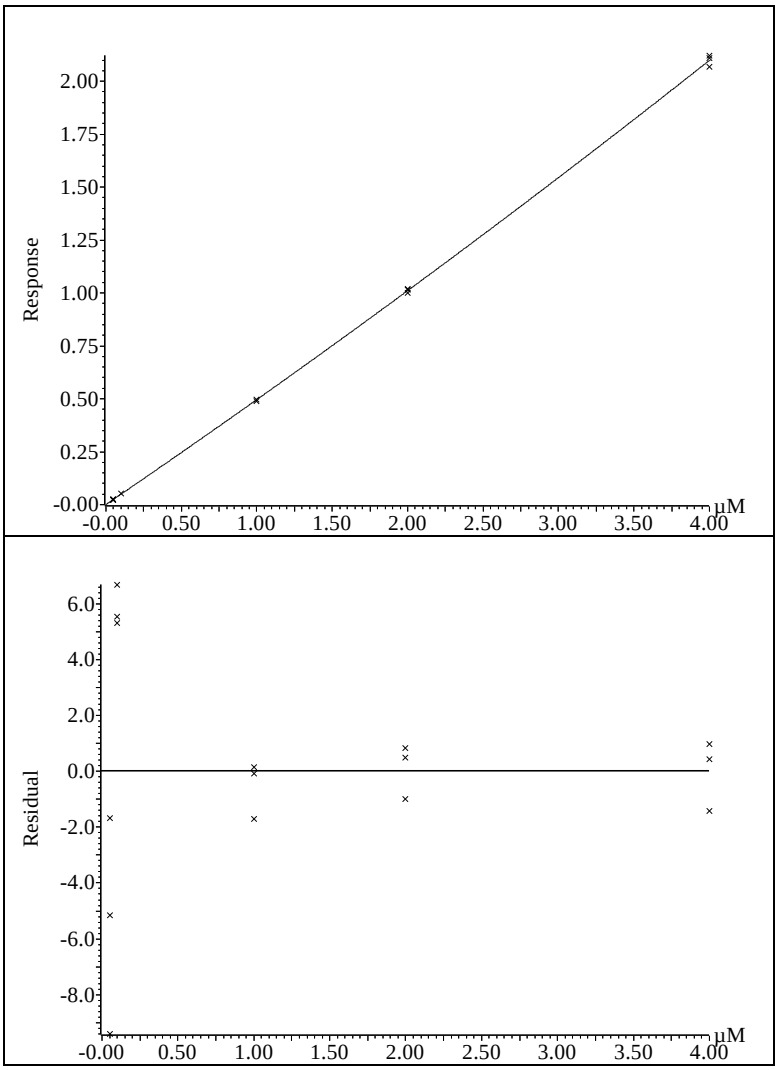

Figure 3 PEth 16:0/18:1 calibration curve (0.05-4.00 µM) and residual plot (percent deviation)

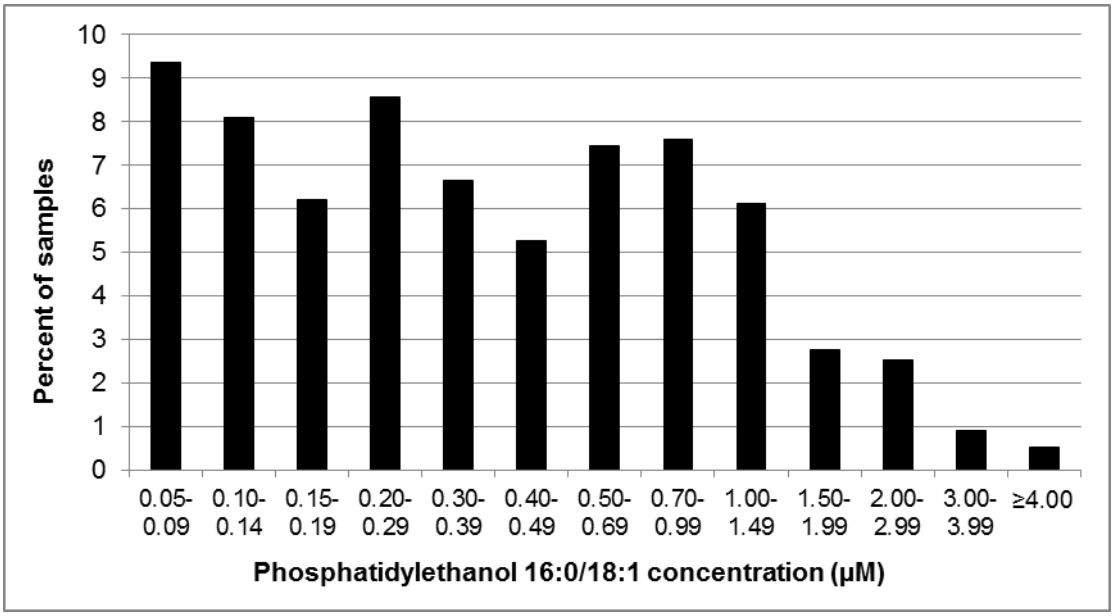

Figure 4 Distribution of the concentrations of PEth 16:0/18:1 in 2286 positive samples (72.1 %) of a total of 3169 consecutive and unselected samples sent to our laboratory for routine analysis.