



Quantification of phosphatidylethanol (PEth) 16:0/18:1 in whole blood with high-resolution accurate mass spectrometry for clinical research

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Keywords

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Application benefits

- Fast determination and quantitation of phosphatidylethanol 16:0/18:1 in whole blood
- Minimal offline sample preparation with whole blood protein precipitation

Goal

Implementation of an analytical method for the quantification of Phosphatidylethanol (PEth) 16:0/18:1 in human whole blood using a Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap™ high-resolution accurate mass (HRAM) spectrometer.

Introduction

Phosphatidylethanol (PEth) is a known alcohol biomarker that exhibits higher clinical sensitivity and specificity than other commonly used alcohol markers. PEth is a group of phospholipids formed through enzymatic reaction between ethanol and phosphatidylcholine. PEth-16:0/18:1 (palmiotic acid/oleic acid) is the predominant molecule extracted from human erythrocytes and can be measured in whole blood as a specific biomarker of alcohol consumption with a detection window of up to 3–4 weeks. In the past, PEth was considered a biomarker for high and sustained alcohol consumption; however, with sensitive LC-MS aided by Orbitrap technology, using this clinical research method, it is now possible to use PEth concentration in blood to differentiate chronic drinking from social drinking or as a marker of absolute abstinence.

In this report, an analytical method for clinical research for the quantification of PEth 16:0/18:1 in human whole blood is reported, while ensuring chromatographic separation of the PEth 16:0/18:1 from isobaric interferences. Sample extraction was performed by protein precipitation of whole blood followed by direct injection of the supernatant onto a Thermo Scientific™ UltiMate™ 3000 RSLC system. A Q Exactive Orbitrap mass spectrometer with heated electrospray ionization was used for detection by selected ion monitoring (SIM) using PEth-d5 as the internal standard.

Experimental

Target analytes

The chemical structure of PEth 16:0/18:1 is reported in Figure 1.

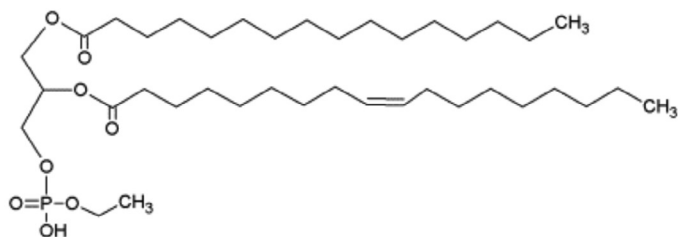


Figure 1. Phosphatidylethanol 16:0/18:1

Sample preparation

Calibrators at 0.03, 0.05, 0.15, 0.5, 1.5, and 5.0 $\mu\text{mol/L}$ and quality control (QC) samples at 0.05 and 0.3 $\mu\text{mol/L}$ were prepared by spiking blank human whole blood

with known amounts of PEth standard solutions in isopropanol. Isopropanol (400 μL) containing the internal standard (PEth-d5) was added to 100 μL of samples, calibrators, and control samples. The samples were shaken vigorously for 10 minutes and centrifuged at 10,000 g for 10 minutes; the supernatant was transferred to clean HPLC vials.

Donor samples

Donor samples were used for investigation of selectivity.

Liquid chromatography

Liquid chromatography was performed on an UltiMate 3000 RSLC system using the following mobile phases:

- Mobile phase A: 10 mmol/L ammonium acetate + 0.1% acetic acid in water
- Mobile phase B: 20 mL of 500 mmol/L ammonium acetate in water and 1 mL acetic acid is added to 750 mL acetonitrile and 250 mL isopropanol
- Mobile phase C: 20 mL of 500 mmol/L ammonium acetate in water and 1 mL acetic acid added to 250 mL acetonitrile and 750 mL isopropanol

Chromatographic separation was achieved on a 50 \times 2.1 mm (1.9 μm) Thermo Scientific™ Hypersil GOLD™ analytical column (P/N 25002-052130) operated using a flow rate of 0.6 mL/min. The gradient profile is described in Table 1. An injection volume of 2 μL was used.

Table 1. LC method description

Time (min)	Flow rate (mL/min)	%A	%B	%C	Divert Valve
0	0.6	10	90	0	Waste
1	0.6	10	90	0	MS
1	0.6	0	0	100	MS
1.5	0.6	0	0	100	MS
1.5	1	0	0	100	Waste
2.1	1	0	0	100	MS
2.5	1	0	0	100	Waste
2.5	0.6	10	90	0	Waste
3.9	0.6	10	90	0	Waste

Mass spectrometry

Detection was performed in SIM acquisition mode on a Q Exactive mass spectrometer with heated electrospray ionization operated in negative ion mode. The ion source conditions and mass spectrometry settings are reported in Table 2 and Table 3, respectively.

Table 2. Ion source settings

Sheath gas flow rate:	40 AU
Aux gas flow rate:	5 AU
Sweep gas flow rate:	2 AU
Spray voltage:	-4500 V
Capillary temperature:	320 °C
Aux gas heater temperature:	400 °C
S-Lens RF level:	100

Table 3. Mass spectrometer settings

Resolution:	70,000
Scan range:	700–710 <i>m/z</i>
AGC target:	2e5
Maximum IT:	250 ms
Microscans:	1
Spectrum data type	Profile

Method evaluation

The method performance was evaluated in terms of limit of quantification, linearity, accuracy, and intra- and inter-assay precision. The analytical accuracy was evaluated by analyzing blank blood samples spiked with known amounts of PEth. Linearity was investigated by back-

calculation of calibration standards. Intra-assay precision was evaluated in terms of percentage coefficient of variation (%CV) using QC samples (n=2) prepared and analyzed in one batch. Inter-assay precision was evaluated on the same QC samples (n=2) prepared and analyzed on four different days. Selectivity was evaluated by analysis of four blank samples from donors who had no intake of alcohol for at least three weeks. Internal standard stability over a batch was investigated by running a batch of donor samples. Matrix effects were evaluated by comparing the response of the internal standard in pure solvent (IPA) to the mean response of the internal standard in whole blood over an analytical run.

Data analysis

Data were acquired and processed using Thermo Scientific™ TraceFinder™ software.

Results and discussion

The analytical method proved to be linear in the calibration range covered by the spiked calibration samples with a lower limit of quantification (LLOQ) of 0.03 µmol/L and an upper limit (ULOQ) of 5.0 µmol/L. The results from back-calculated calibration standards are presented in Table 4 and the calibration curve is presented in Figure 2. Representative chromatograms at the LOQ (0.03 µmol/L) and at 0.05 µmol/L (proposed cutoff level) for both the analyte and the internal standard are presented in Figures 3 and 4, respectively.

Intra- and inter-assay accuracy and precision results are presented in Table 5. Both intra- and inter-assay accuracy and precision were well within acceptance limits (±15%) at all tested levels.

Table 4. Back-calculated concentrations for the calibration standards

	Nominal Conc. (µM)	Calculated Conc. (µM)				Mean	Mean Accuracy %
		Day 1	Day 2	Day 3	Day 4		
Cal1	0.03	0.029	0.033	0.032	0.029	0.031	103
Cal2	0.05	0.054	0.042	0.046	0.052	0.049	97
Cal3	0.15	0.148	0.147	0.131	0.157	0.146	97
Cal4	0.5	0.497	0.515	0.481	0.511	0.501	100
Cal5	1.5	1.326	1.485	1.574	1.499	1.471	98
Cal6	5.0	5.505	5.304	5.605	4.62	5.259	105

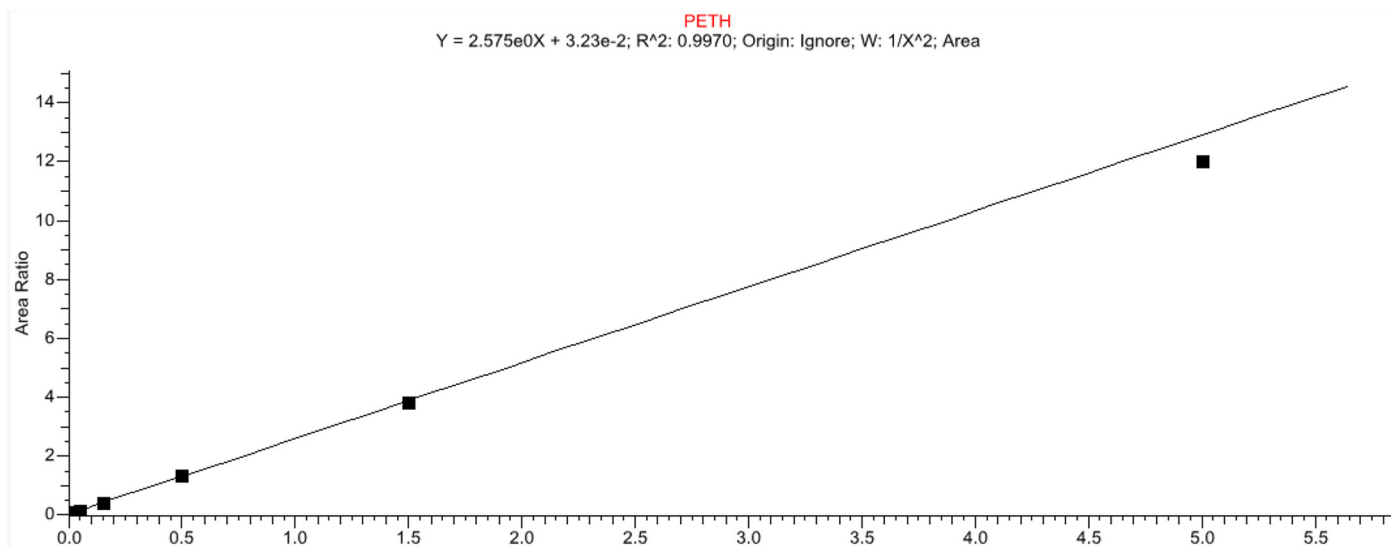


Figure 2. A representative calibration curve with $1/x^2$ weighting

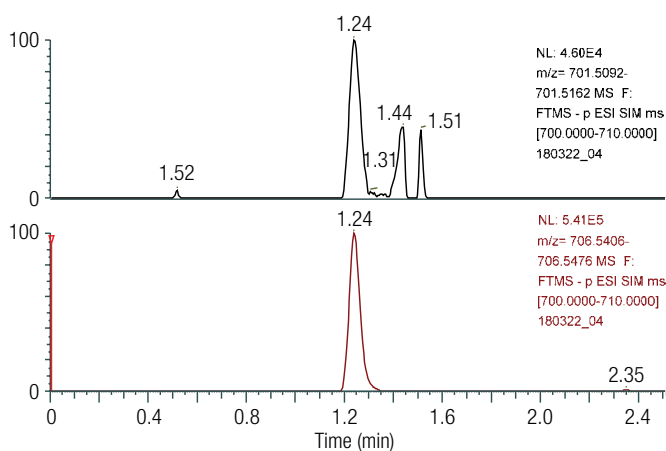


Figure 3. A representative chromatogram from a LLOQ (0.03 $\mu\text{mol/L}$) sample, extracted using a mass accuracy window of ± 5 ppm, showing the analyte and the internal standard

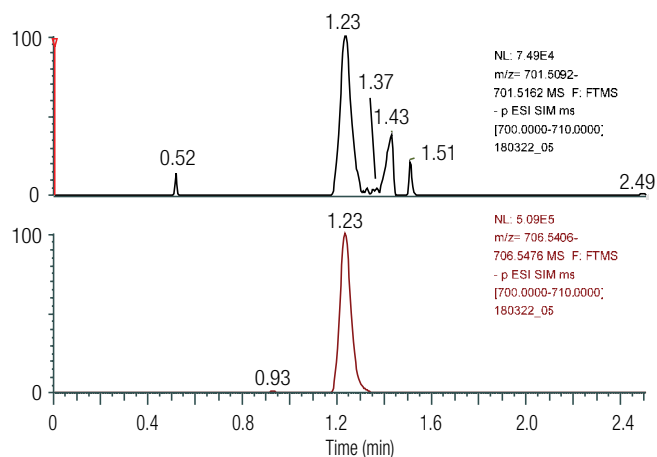


Figure 4. A representative chromatogram from a calibration sample at 0.05 $\mu\text{mol/L}$ (proposed cutoff level), extracted using a mass accuracy window of ± 5 ppm, showing the analyte and the internal standard

Table 5. Intra- and inter-assay accuracy and precision results

	QC 1				QC 2			
	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4
Nominal conc. ($\mu\text{mol/L}$)	0.050				0.300			
Replicate 1	0.050	0.057	0.046	0.052	0.281	0.332	0.293	0.327
Replicate 2	0.051	0.048	0.045	0.046	0.287	0.313	0.285	0.324
Mean calc conc ($\mu\text{mol/L}$)	0.051	0.053	0.046	0.049	0.284	0.323	0.289	0.326
Intra-day accuracy (%)	101	105	91	98	95	108	96	109
Intra-day precision (%)	1.4	12.7	1.4	8.5	1.4	4.5	1.9	0.7
Overall calc conc ($\mu\text{mol/L}$)	0.049				0.305			
Inter-day accuracy (%)	98.8				101.8			
Inter-day precision (%)	8.0				7.0			

The results from the investigation of selectivity are presented in Figure 5. Only small peaks with integrated areas $\ll 20\%$ of the area at LLOQ were found.

The method offered an excellent internal standard stability, with a %CV over 65 injections of 5.1%. Results are graphically reported in Figure 6.

180305_002 PETH m/z: 701.5127 180308_002 PETH m/z: 701.5127 180322_02 PETH m/z: 701.5127 180322_71 PETH m/z: 701.5127

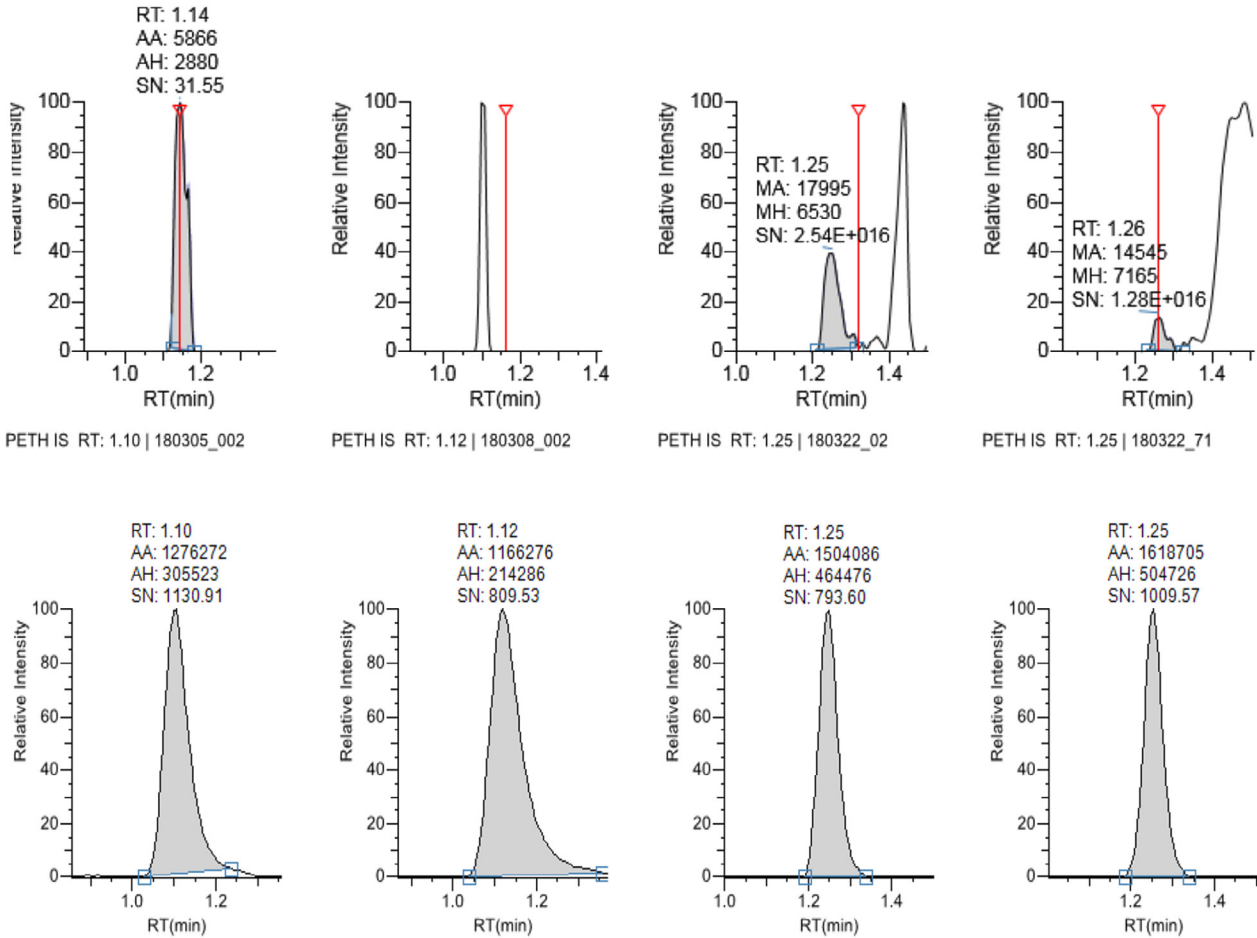


Figure 5. Chromatograms of four blank samples and internal standards

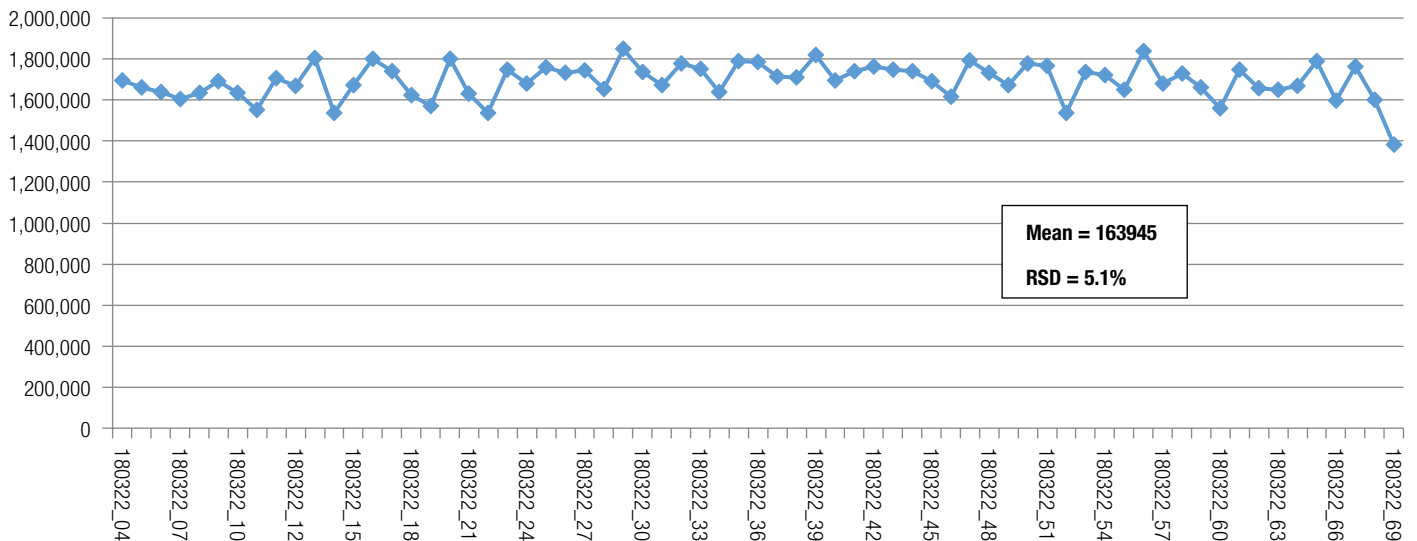


Figure 6. Investigation of internal standard stability

Table 6. Investigation of matrix effects

Sample ID	Day 1	Day 2	Day 3	Day 4
Solvent Blank 1	1231588	962149	1353233	1234783
Solvent Blank 2	1273486	1110837	1372335	1291963
Mean	1252537	1036493	1362784	1263373
Mean IS response	1497131	1240765	1445711	1693945
Matrix effect %	120	120	106	134
Average	120			

The results from the investigation of matrix effects are presented in Table 6. A positive (enhancement) effect of about 20% was observed.

Conclusions

A liquid chromatography coupled to a high-resolution accurate mass spectrometer was used to develop a robust, reliable, and sensitive analytical method for clinical research for the quantification of phosphatidylethanol 16:0/18:1 in human blood. The method was implemented and analytically validated using an UltiMate 3000 RSLC system coupled to a

Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometer. This analytical method allows for the chromatographic separation of PEth 16:0/18:1 from interfering isobaric species, ensuring accurate quantitation of PEth using a simple sample preparation based on protein precipitation. The method meets research laboratory requirements for sensitivity, linearity, accuracy, and precision in the range 0.03–5.0 µmol/L.

It is possible to run this method on a Thermo Scientific™ Q Exactive™ Focus™ Hybrid Quadrupole-Orbitrap mass spectrometer.

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