POSTER NOTE

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A Method for the Quantification of PEth 16:0/18:1 in Human Blood based on UHPLC and Orbitrap High Resolution Accurate Mass (HRAM) Spectrometry

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ABSTRACT

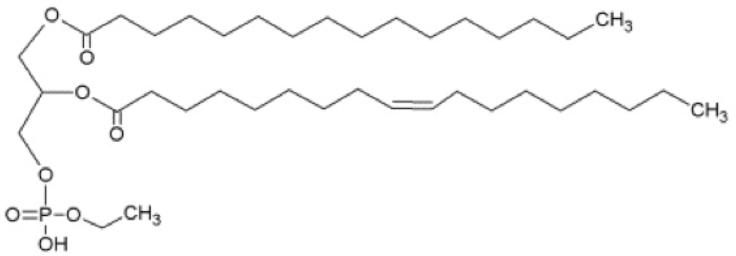
Phosphatidylethanol (PEth) is an abnormal phospholipid that is found in red blood cells membrane after intake of ethanol. PEth 16:0/18:1 is the most abundant form and the precence of this molecule is used as an alcohol biomarker in the Swedish Healthcare system.

This work describes an LC-MS method for clinical research measurements of PEth 16:0/18:1 in human blood and the results from the validation of this method. Mass spectrometric detection was performed by monitoring of the intact mass on a Thermo Scientific™ Exactive Plus™ high resolution mass spectrometer using heated electrospray ionization. The limit of quantification was 0.03 µmol/L, cutoff was 0.05 µmol/L and the upper limit of quantification was 5.0 µmol/L. Accuracy for the method was measured at 0.05 and 0.30 µmol/L in terms of percent bias (% Bias) between nominal and experimental concentration and was always within ± 15%. Precision at the same concentrations calculated as percentage coefficient of variation (% CV) was always below 15%. This high resolution method was cross-validated against a method based on a triple quadrupole mass spectrometer[1] and excellent agreement between the two was found. Analysis of samples from an international quality control program also showed excellent agreement.

INTRODUCTION

The molecular composition of PEth is complex and with mass spectrometry several different PEth forms have been detected[2]. Common for all PEth forms is a glycerol head group with two hydrocarbon side chain that varies in both number of carbon and saturation. Of these, PEth 16:0/18:1 is the most abundant form and is used in the laboratory to detect alcohol intake (Figure1). Hence, a method for the determination of PEth 16:0/18:1 in human blood has been developed. The method is based on UHPLC and monitoring of the intact molecular ion on a High Resolution Accurate Mass Spectrometer (UHPLC-HRAM-MS).

Figure 1. PEth 16:0/18:1



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MATERIALS AND METHODS

Sample preparation

100 µL human whole blood, haemolyzed by freezing at -70ºC, (specimens, calibrators and control samples) was pipetted into 1.5 mL Eppendorf vials. 400 µL isopropanol was added and the sample was shaken vigorously for 10 minutes. The sample was centrifuged at 10000xg for 10 minutes and the supernatant was transferred to clean vials.

Calibration and QC-samples

Calibration samples and QC-samples were prepared by spiking known amount of pure standard PEth 16:0/18:1 in an isopropanol solution into haemolysed PEth negative blood samples. This procedure was chosen for practical reasons and has shown to give the same results as spiking fresh whole blood. Calibration samples were prepared at concentrations of 0.03, 0.05, 0.15, 0.50, 1.5 and 5.0 µmol/L and QC-samples were prepared at 0.05 and 0.30 µmol/L. A deuterated analog of PEth with 5 deuteriums was used as internal standard.

Liquid Chromatography

Separation was performed on a Thermo Scientific™ UltiMate ™ 3000 RSLC UHPLC system consisting of a HPG pump, an autosampler, a degasser and a column oven using an isocratic mobile phase. A wash step using a stronger mobile phase was used to clean the column between injections.

Column: Thermo Scientific™ Hypersil Gold™ C_{18} 2.1x50 mm, 1.9 µm particles

Mobile Phases:

A: 10 mM ammonium acetate in water + 0.1% glacial acetic acid

B: Acetonitrile / Isopropanol / Water 75 / 25 / 2 + 10mM ammonium acetate and 0.1% glacial acetic acid

D: Acetonitrile / Isopropanol / Water 25 / 75 / 2 + 10mM ammonium Acetate and 0.1% glacial acetic acid

Injection volume:	2 µL
Column Temperature:	60ºC
Autosampler temperature:	10ºC

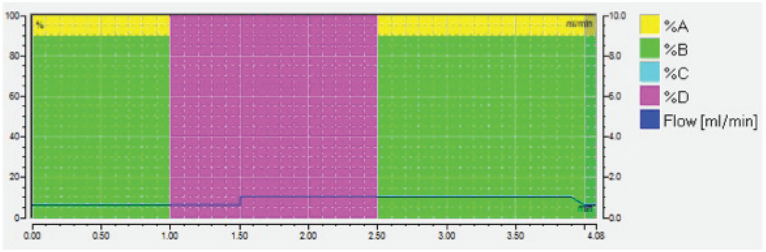
Autosampler wash solution: Magic mix (Acetonitrile / isopropanol / Acetone 45/45/10)

A switch valve was used to divert the flow to waste before 0.5 minutes and after 1.5 minutes.

Table 1: Mobile Phase Composition

Retention time (min)	Flow (mL/ min)	%A	%B	%D
0.0	0.6	10	90	0
1.0	0.6	10	90	0
1.0	0.6	0	0	100
1.5	0.6	0	0	100
1.5	1.0	0	0	100
2.5	1.0	0	0	100
2.5	1.0	10	90	0
3.9	1.0	10	90	0
4.0	0.6	10	90	0

Figure 2. Mobile Phase Composition



Mass Spectrometry

Samples were detected using an Exactive Plus high resolution accurate mass (HRAM) spectrometer. Data was collected from 650-750 amu in Negative mode using a heated electrospray (HESI II) ion source.

Mass Spectrometer settings

Mass range:	650-750 amu
Resolution@200:	70000
AGC Target:	3E6
Max Injection Time:	250

Ion Source settings:

Sheath Gas Flow Rate:	40
Aux Gas flow Rate:	5
Sweep Gas Flow Rate:	5
Spray Voltage:	5 kV
Capillary Temp:	320C
S Lens RF Level:	100
Vaporizer temp:	450C

Data Analysis

Data collection and analysis was performed using Thermo Scientific™ TraceFinder ™ 3.3 software. Linear calibration based on area response ratio with a $1/x^2$ weighting was used.

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RESULTS

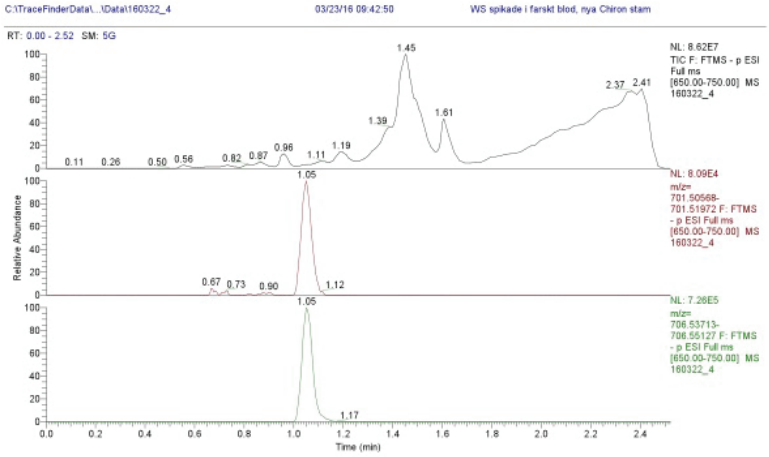
Selectivity

Selectivity was obtained by the selective detection using the HRAM mass spectrometer with a resolution setting of 70000 and by chromatographic separation of isobaric compounds. Chromatograms were extracted from the full scan data at ±10 ppm from the molecular ions of the parent masses of PEth and the internal standard respectively, as described in Table 2. Chromatographic separation was obtained using a mobile phase consisting of acetonitrile, isopropanol, ammonium acetate and a C_{18} stationary phase. A chromatogram of the lowest standard (0.03 µmol/L) is shown in Figure 3.

Table 2. Ranges for extraction of chromatograms from full scan data

Compound	Chemical Formula	Species	m/z	XIC Range ± 10 ppm
PEth	$C_{39}H_{75}O_8P$	M-H	701.5127	701.5057-701.5197
PEth D5	$C_{39}H_{70}D_5O_8P$	M-H	706.5441	706.5370-706.5512

Figure 3. Chromatogram of lowest standard (0.03 µmol/L)

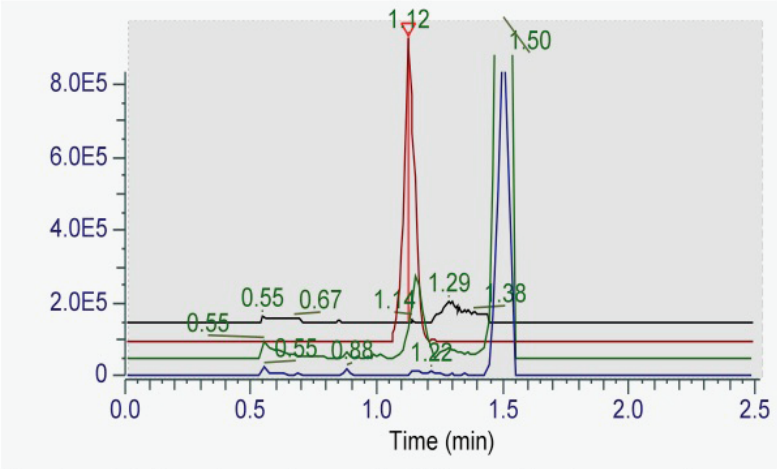


Chromatogram of the lowest calibration standard (0.03 µmol/L) showing the Total Ion Current in the upper trace, PEth 16:0/18:1 in the middle trace and the internal standard in the lower trace.

The selectivity was evaluated by running blank samples, i.e. donor samples from people who had not had any intake of alcohol for three weeks. None of the tested samples had a signal that was higher than 20% of the signal at LLOQ at the retention time of PEth.

The selectivity was further evaluated using a Q Exactive mass spectrometer coupled to the same LC-system as the Exactive Plus system. Full Scan data was compared to PRM data where parent ions were isolated using a 1 amu window and chromatograms were extracted for the fragments 281.2490 (quan ion) and 255.2331 (qual ion). A PEth negative sample was injected and as shown in Figure 4 there was no interference in the full scan trace, while for the PRM detection there was a small interference in the qual ion trace and a quite large interference in the quan ion trace.

Figure 4. Chromatogram of a PEth negative donor sample run in simultaneous full scan and PRM on a Q Exactive mass spectrometer



Overlay plot of the chromatogram of a PEth negative donor sample showing PEth intact parent ion in full scan in the upper trace (black), PEth IS in the 2nd trace (purple), PRM quan ion fragment in the 3rd trace (green) and the qual ion fragment in the lower trace (blue).

Linearity

Linearity was investigated by evaluation of the backcalculated accuracies of the calibration standards from the validation batches. The results are presented in table 4.

Table 4: Accuracies of backcalculated calibration standards

Batch no / Nominal conc. (µmol/L)	1	2	3	4
0.03	100	100	100	87
0.05	98	102	98	96
0.15	101	99	100	95
0.5	100	96	100	98
1.5	108	99	100	97
5	91	105	101	112

Accuracy and Precision

Accuracy and precision was determined at two levels, the cutoff level at 0.05 µmol/L and at 0.30 µmol/L, on four different ocasions. The results are presented in table 2 and table 3.

Table 2: Intra assay accuracy and precision

Batch no	1	2	3	4
Nominal Conc. (µmol/L)	**0.050**	**0.050**	**0.050**	**0.050**
1	0.049	0.052	0.050	0.045
2	0.049	0.053	0.049	0.044
3	0.050	0.052	0.049	0.043
4	0.049	0.052	0.051	0.045
5	0.049	0.053	0.052	0.047
Mean	**0.049**	**0.052**	**0.050**	**0.045**
Mean Accuracy	**98**	**105**	**100**	**90**
CV%	**0.9**	**1.0**	**2.6**	**3.3**
Batch no	**1**	**2**	**3**	**4**
Nominal Conc. (µmol/L)	**0.300**	**0.300**	**0.300**	**0.300**
1	0.301	0.313	0.296	0.303
2	0.301	0.309	0.297	0.303
3	0.304	0.313	0.289	0.302
4	0.305	0.313	0.295	0.298
5	0.305	0.321	0.313	0.310
Mean	**0.303**	**0.314**	**0.298**	**0.303**
Mean Accuracy	**101**	**105**	**99**	**101**
CV%	**0.7**	**1.4**	**3.0**	**1.4**

Table 3: Inter assay accuracy and precision (n=20)

Nominal conc. (µmol/L)	0.050	0.300
Mean Accuracy	98	102
CV%	6.1	2.6

Quality Control program

Samples from an international quality control program were analyzed at 5 occasions. The results are presented in table 5.

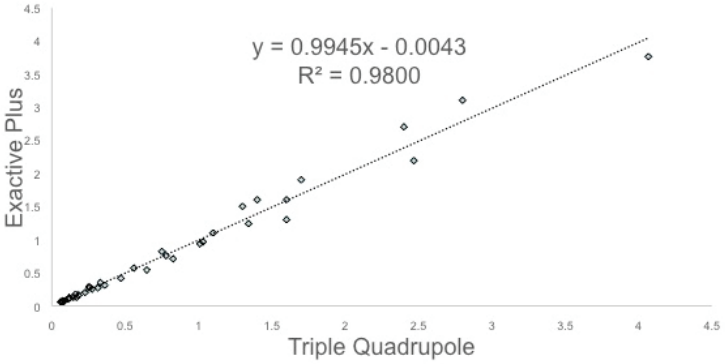
Table 5. Accuracies after analysis of samples in a national quality control program.

Nominal Conc. (µmol/L)	1	2	3	4	Mean Accuracy %	CV%
0.01	<0.03 in all samples				N/A	N/A
0.09	99	101	108	110	104	5.3
1.26	103	96	97	96	98	3.1

Cross validation

The results from this HRAM method were compared with results from a method based on a Triple Quadrupole[1] at another Swedish hospital, which is being used for routine measurements of PEth. The results are shown in Figure 5.

Figure 5. Comparison of results from Exactive Plus method and a method based on triple quadrupole



DISCUSSION

In blood there are several species with the same elemental composition as PEth 16:0/18:1. There are also species which have parent ions with similar masses that produce the same fragments as PEth 16:0/18:1. As always for analytical methods in complex biological matrices, selectivity is critical. In LC-MS the selectivity is usually mainly derived from the mass spectrometer, with some support of a chromatographic separation. For analysis of PEth 16:0/18:1 chromatographic separation is critical, no matter if the HRAM approach or a fragmentation approach is used. For the presented chromatographic system selectivity was shown to better using HRAM than using a fragmentation approach.

CONCLUSIONS

A method for the determination of PEth 16:0/18:1 in whole blood using HRAM measurement of the intact molecular ion is presented. The validation shows that the method is

- Selective, HRAM superior to fragmentation
- Linear in the range 0.03-5.0 µmol/L
- Accurate, well within bioanalytical guidance limits[3]
- Reproducible, precision well within bioanalytical guidance limits[3]
- Analysis of samples from an international quality control program showed excellent agreement
- The methods showed excellent correlation (R^2=0.9800) to a method used for routine PEth measurements at another Swedish hospital

REFERENCES

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