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Method development for routine liquid chromatography–mass spectrometry measurement of the alcohol biomarker phosphatidylethanol (PEth) in blood

Yufang Zheng [a], Olof Beck [b], Anders Helander [a,*]

[a] Alcohol Laboratory, Department of Laboratory Medicine, Karolinska Institutet, Stockholm, Sweden
[b] Department of Medicine, Karolinska Institutet, Stockholm, Sweden

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ABSTRACT

Background: Phosphatidylethanol (PEth) is a group of phospholipids formed from ethanol and phosphatidylcholine by action of phospholipase D. Measurement of PEth in whole blood samples is employed as an alcohol biomarker. This work aimed to further develop an LC–MS method for PEth to make it practical for routine laboratory use.
Methods: Blood samples were obtained from blood donors and from the clinical samples pool. A whole blood total lipid extract was separated on a C4 column, followed by ESI-MS detection of the deprotonated molecules in SIM mode or ESI-MS/MS detection of the major product ions (fatty acid fragments) by SRM.
Results: Initial results indicated that individual calibration curves are required for MS quantitation of some PEth forms, and that deuterated analogs are preferable over phosphatidylpropanol as the internal standard. PEth-16:0/18:1 was the single most sensitive molecular form as alcohol biomarker, being detected in every of 211 blood specimens containing 0.1–20 μmol/L total PEth at reporting limits in the range 0.1–1.0 μmol/L. PEth-16:0/18:1 and 16:0/18:2, accounting for about 36% and 26%, respectively, of the total amount, correlated well with total PEth ($R^2 = 0.922–0.940$), but the correlation was better for the sum of both forms ($R^2 = 0.994$). Based on analysis of specimens from 200 blood donors, 95% reference intervals (CLSI C28-A3) were estimated to be <0.70 μmol/L for total PEth, <0.20 μmol/L for PEth-16:0/18:1, and <0.18 μmol/L for PEth-16:0/18:2.
Conclusions: The LC-ESI-MS(/MS) method allowed for simultaneous qualitative and quantitative measurements of PEth forms in whole blood samples. Related to the routine application of blood PEth as alcohol biomarker, reference intervals were suggested for total PEth and the major molecular forms PEth-16:0/18:1 and 16:0/18:2.

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1. Introduction

An abnormal phospholipid originally discovered in organs of rats exposed to ethanol was identified as phosphatidylethanol (PEth) [1,2]. PEth is formed from ethanol and phosphatidylcholine (PC) in cell membranes by a transphosphatidylation reaction catalyzed by phospholipase D (PLD) [3]. PLD uses water in the hydrolysis reaction that generates phosphatidic acid (PA) and choline. However, because PLD preferentially utilizes ethanol over water, PEth is generated at the expense of PA in the presence of ethanol [4–6]. In humans, PEth was detected in blood samples [7–9] and autopsy material [10] collected from chronic heavy drinkers and measurement of PEth in blood was proposed for use as an alcohol biomarker [7,11–13]. The blood PEth concentration has been demonstrated to correlate with the amount of alcohol consumed, even though the relationship varies considerably between individuals [14–16].

Measurement of PEth was originally done by thin-layer chromatography but this technique is less suitable for quantitative analysis and was later replaced by high-performance liquid chromatography with evaporative light-scattering detection (HPLC-ELSD) [17,18]. Recently, methods based on capillary electrophoresis (CE) coupled to UV [19] or mass spectrometric (MS) [20] detection, and HPLC coupled to MS (LC–MS and LC–MS/MS) detection [21], have been introduced. The methodological progress has greatly increased the interest in blood PEth as a routine alcohol biomarker, as reflected in a growing number of clinical and medico-legal evaluations [14–16,22–28].

Being derived from ethanol and PC, an advantage of PEth as alcohol biomarker over liver function tests such as gamma-glutamyltransferase is its high specificity for alcohol. Nonetheless, because the PLD enzyme is also active at low temperature and even on storage at −20 °C, PEth may be generated post-sampling if ethanol is present [10,21,29,30]. This implies a risk for artifactual formation leading to false indications of prior heavy drinking [11,23].

* Corresponding author at: C1:74, Karolinska University Laboratory Huddinge, SE-141 86 Stockholm, Sweden. Tel.: +46 8 58581293; fax: +46 8 58581260.
E-mail address: anders.helander@ki.se (A. Helander).

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PEth is not a single molecule but a group of phospholipids with a common phosphoethanol head onto which two fatty acids of variable carbon chain length and degree of saturation are attached [21]. Accordingly, when HPLC-ELSD and CE-UV methods, measuring a total amount of PEth, are replaced by selective MS-based methods, enabling identification and quantitation of the individual molecular forms [21,31], it has become necessary to determine which PEth species should be the analytical target. In blood from heavy drinkers, 9 major forms were identified by MS with PEth-16:0/18:1 (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanol) and PEth-16:0/18:2 (1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoethanol) being predominant [21]. Together PEth-16:0/18:1 and 16:0/18:2 accounted for approximately 60% of the total amount. Later on, the presence of 48 PEth forms was indicated in a blood sample from an autopsy case [32].

The aim of the present work was to further develop LC–MS(/MS) for qualitative and quantitative measurements of PEth in human blood [21] and make it practical for routine laboratory use.

2. Materials and methods

2.1. Chemicals

PEth-16:0/18:1 reference material was obtained from Biomol Research Laboratories, PEth-16:0/18:2 (>99% purity; synthesized on special request), PC-16:0/18:1 (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine), PC-16:0/18:2 (1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine) and phosphatidylpropanol (PProp-18:1/18:1) were from Avanti Polar Lipids, phospholipase D (PLD type IV from cabbage) from Sigma Aldrich, and deuterated ethanol (ethanol-d_6) was from Merck. All other chemicals were of analytical or HPLC grade, and the water was of HPLC grade.

2.2. Synthesis of deuterium-labeled PEth as internal standards

Two deuterium-labeled PEth analogs for use as internal standards were synthesized from the corresponding PC, according to an enzymatic procedure with minor modifications [33]. PC-16:0/18:1 or PC-16:0/18:2 was evaporated to dryness and dissolved in diethyl ether, mixed with acetate buffer containing 100 mmol/L $CaCl_2$, and ethanol-d_6 was added. The reaction was started by addition of PLD. Following 1.5 h incubation under gentle mixing at 30 °C, the reaction was stopped by addition of acetonitrile and the solution was evaporated to dryness under a stream of nitrogen gas. The residue was dissolved in a solution of 2 mmol/L ammonium acetate and 80% acetonitrile, centrifuged, and the protein pellet removed. The concentration was determined by comparison with the unlabeled substances. Initially, also PEth-16:0/18:2 reference material was synthesized from PC-16:0/18:2 and ethanol using this procedure.

The deuterium-labeled materials were characterized by LC–MS/MS using flow injection infusion. PEth-16:0/18:1 has a molecular mass of *m/z* 701.5 in negative ion mode $[M-H]^-$, whereas the labeled product showed a *m/z* 706.5, i.e. being penta-deuterated. In product ion scan, the spectrum pattern was very similar to that of PEth-16:0/18:1 with *m/z* 281.1 (18:1 fatty acid) and *m/z* 255.3 (16:0 fatty acid) as the dominant product ions. Corresponding results were obtained for the synthesis of deuterium-labeled PEth-16:0/18:2. The deuterated and non-deuterated forms showed very similar retention times. Taken together, these results confirmed that the deuterium labeling was located to the ethyl head-group of PEth. Both penta-deuterated PEth materials (PEth-16:0/18:1-d_5 and 16:0/18:2-d_5) contained approximately 1% impurity of the corresponding unlabeled form. Therefore, in routine use, the amount of internal standard was adjusted so that the impurities were well below the lower quantitation limit (LLOQ) of the method.

2.3. Stock solutions

Stock solutions of PEth reference materials (14.0 μmol/L of each), and of deuterium-labeled PEth (0.77 μmol/L) and PProp (1.35 μmol/L) used as internal standards, were prepared in a 1:5 solution of 2 mmol/L ammonium acetate and acetonitrile and stored at −20 °C until use. Under these conditions, the stock solutions were stable for at least 6 months [21].

2.4. Blood samples

The blood specimens used for method development were de-identified surplus volumes from blood donors with unknown alcohol consumption, and from the clinical samples pool at the Karolinska University Hospital (Stockholm, Sweden). The blood was collected in EDTA tubes and stored at 4 °C for <3 days before analysis. The PEth concentration is stable for at least 3 weeks in blood samples stored refrigerated [18]. The procedures followed were approved by the ethics committee at the Karolinska University Hospital.

2.5. Sample preparation

Total lipid extracts of whole blood samples were prepared as previously described [21], using stepwise addition of 100 μL blood to 600 μL isopropanol and 50 μL (PProp stock solution) or 100 μL (PEth-16:0/18:1-d_5 plus PEth-16:0/18:2-d_5 stock solutions) internal standard solution under constant vortex-mixing. Thereafter, the samples were gently mixed for 10 min and 2×450 μL heptane was added with mixing after each addition. The samples were centrifuged for 10 min at 2000 *g* at 4 °C. The clear supernatants were transferred to new glass tubes and evaporated to dryness under a stream of nitrogen gas at 30 °C using a metal block. The dried extracts were dissolved in 50 μL heptane, followed by addition of 50 μL acetonitrile and 75 μL isopropanol, and finally transferred to 0.3-mL glass autosampler vials and centrifuged for 10 min at 1000 *g*.

Standards and quality controls were prepared by adding 100 μL PEth reference material in freshly made diluted stock solutions to the isopropanol before mixing with 100 μL of PEth-negative blood.

2.6. Single MS analysis of PEth

LC–MS quantitation of different PEth species was performed by an electrospray ionization (ESI) method on an Agilent 1100 series LC–MS instrument, as previously described [21]. ESI-MS analysis was performed using selected ion monitoring (SIM) in negative mode of the deprotonated molecules (Fig. 1A). The MS conditions were: drying gas flow 10.0 psi, nebulizer gas 20 psi, drying gas temperature 350 °C, and capillary voltage 3000 V. Chromatographic separation of the blood lipid extracts was achieved on a 5-μm HyPurity C4 column (Thermo Scientific) maintained at 25 °C. The LC system was operated in gradient mode with solvent A being 20% 2 mmol/L ammonium acetate and 80% acetonitrile and solvent B being 100% isopropanol. From sample injection until 2.0 min, isocratic elution with 90% A and 10% B was used; from 2.0 to 3.0 min, a linear gradient to 50% B; from 3.0 to 6.0 min, a linear gradient to 100% B; from 6.0 to 7.0 min, isocratic elution with 100% B; and from 7.0 to 8.0 min, a linear gradient back to 90% A and 10% B. Thereafter, the column was equilibrated for 10 min prior to the next injection. The flow rate was 200 μL/min and the sample injection volume 10 μL.

2.7. Tandem MS analysis of PEth

The LC tandem-MS system was a Perkin-Elmer series 200 LC system connected to Sciex API 2000MS with the ESI interface operated in negative ion mode, and Analyst 1.1 software (Applied Biosystems). The chromatographic conditions were identical to those employed for single MS analysis. ESI-MS/MS analysis was performed using selected

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