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Analytical Biochemistry 341 (2005) 83–88
ANALYTICAL BIOCHEMISTRY
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A method for determination of phosphatidylethanol from high density lipoproteins by reversed-phase HPLC with TOF–MS detection

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Received 3 January 2005
Available online 17 March 2005

Abstract

Phosphatidylethanol (PEth) is a unique phospholipid that is formed in the body only in the presence of ethanol. According to a new hypothesis, blood high-density lipoprotein (HDL) particles may act as carriers of PEth and mediate part of the antiatherogenic effects of moderate alcohol drinking. Liquid chromatographic method using reversed-phase C8 column and negative ion mode electrospray ionization–mass spectrometry detection with time-of-flight (TOF) instrument was developed for the determination of very small amounts of PEth that might be present on blood HDL particles. The samples used in the current study were human HDL spiked with PEth and internal standard phosphatidylpropanol (PProp). The use of reversed-phase column enabled a short analysis time of 19 min/injection, which is only one-third of the earlier normal-phase methods reported. Because of the narrow bore column (2.1 mm i.d.) and short analysis time, the solvent consumption was decreased. The sensitivity of detection obtained with TOF–MS was better than that of previous methods, with the detection limit being as low as 1 ng/ml in injected sample (20 pg on-column ~28 fmol PEth), corresponding to approximately 6.7 ng of PEth in milliliter of unprepared HDL. Good linearity of detection was obtained for a range of 1–100 ng/ml of PEth, whereas all of the deviations in precision and accuracy were less than 15%.

Keywords: Ethanol; High-density lipoproteins; Phosphatidylethanol; Reversed-phase HPLC; LC/MS

Light to moderate alcohol consumption is associated with a reduced risk of coronary heart disease. An increase in high-density lipoprotein (HDL)[1] cholesterol concentration and a decrease in low-density lipoprotein (LDL) cholesterol concentration are well-known antiatherogenic effects of alcohol intake. There are, however, also other mechanisms by which alcohol drinking protects from atherosclerosis. A new hypothesis suggests that part of the protection is mediated by lipoprotein particles acting as carriers of phosphatidylethanol [1] (PEth, Fig. 1), which is an abnormal phospholipid formed in the cell membranes only in the presence of ethanol [2,3]. The reaction between phosphatidylcholine and ethanol is catalyzed by phospholipase D (PLD) [4,5]. PLD activity has been detected in a number of mammalian tissues, including brain, liver, erythrocytes, and neutrophil granulocytes [6–9]. Because the elimination of PEth from the body is slow, detectable levels of PEth can be found from the blood even 14 days after the administration of ethanol [10]. Because of the specific formation and slow disappearance of PEth from the body, it has

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[1] *Abbreviations used:* HDL, high-density lipoprotein; LDL, low-density lipoprotein; PEth, phosphatidylethanol; PLD, phospholipase D; HPLC, high-performance liquid chromatography; ELSD, evaporative light-scattering detector; MS, mass spectrometry; RP, reversed-phase; ESI–MS, electrospray ionization–mass spectrometry; PProp, phosphatidylpropanol; PBS, phosphate-buffered saline; CID, collision-induced dissociation; LLQ, lowest limit of quantification.

0003-2697/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2005.03.001

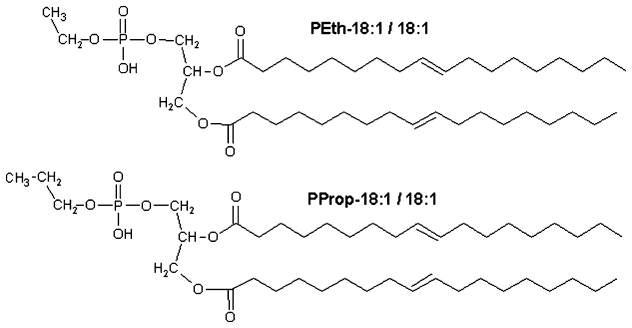

Fig. 1. Structures of phosphatidylethanol (PEth) and phosphatidylpropanol (PProp).

been suggested that PEth could be used as a marker of alcohol abuse [10,11].

Few articles have been published concerning the high-performance liquid chromatography (HPLC) analysis of PEth from blood [12,13] and rat heart [14,15] using normal-phase columns with hexane- and 1-propanol-based gradient elution. The long equilibration times of these chromatographic systems have led to HPLC run times lasting 1 h or longer and, with the flow rates used (0.4–1.0 ml/min), also to very high solvent consumption. The acquisition of the data with the methods has been carried out using evaporative light-scattering detectors (ELSDs) [12–14], mass spectrometry (MS) [14], or fluorescence detectors [15]. The detection limits obtained with ELSDs vary in the range of 4–6 μg/ml (125–200 ng PEth on-column), whereas much lower detection limits of 9 ng/ml (450 pg on-column) and 5 ng/ml (100 pg on-column) have been obtained with fluorescence and MS detection, respectively.

Our aim was to develop a faster analysis method and to optimize the sensitivity of detection for very low PEth concentrations that might be present in HDL particles, as suggested by the above hypothesis. Because the reversed-phase (RP) columns need much shorter column equilibration times between consecutive gradient elution runs, and the elution solvents used with RP–HPLC are more suitable (than those used with normal-phase HPLC) for interfacing chromatographic with electrospray ionization–mass spectrometry (ESI–MS), we decided to develop a method based on RP column.

Materials and methods

Chemicals

HPLC-grade acetonitrile and isopropanol were purchased from Merck (LiChrosolv, Darmstadt, Germany). HPLC-grade hexane was purchased from LabScan (Dublin, Ireland). Laboratory water was purified with a Simplicity 185 water purifier (Millipore, Molsheim, France). Ammonium acetate was obtained from BDH Laboratory Supplies (Poole, UK). The PEth (18:1/18:1) and phosphatidylpropanol (PProp, 18:1/18:1) standards were obtained from Avanti Polar Lipids (Alabaster, AL, USA).

Lipoprotein isolation

HDLs were isolated from the plasma of healthy volunteers by sequential ultracentrifugation as described previously [16]. The volunteers had abstained from alcohol for several days before the collection of blood samples. HDL particles were isolated at a density of 1.063–1.210 g/ml. After ultracentrifugation, the lipoproteins were dialyzed against phosphate-buffered saline (PBS) at 4 °C overnight. Protein and phospholipid concentrations in HDL were determined as described previously [1].

Sample preparation and extraction

HDL particles (15 μl, with protein and phospholipid concentrations being 16.4 and 9.0 mg/ml, respectively) were extracted with 200 μl of hexane-2-propanol containing the internal standard (3:2, v/v). After centrifugation at 20,800*g* at room temperature for 10 min, supernatant was removed and transferred to a new Eppendorf tube and solvent was evaporated in a vacuum dryer. The samples were redissolved in 100 μl of water-2-propanol-acetonitrile (1:1:3, v/v). The samples used in method development were prepared similarly from PEth-free HDL and were spiked with PEth.

LC–MS

A Waters 2695 Alliance HPLC system (Waters, Milford, MA, USA) was used. The HPLC separation was performed with a Waters Symmetry C8 2.1 × 100-mm column with a 3.5-μm particle size (Waters) at 30 °C. The injection volume was 20 μl. The HPLC eluents were aqueous 2 mM ammonium acetate (A), acetonitrile (B), and isopropanol (C). Isocratic elution with 20% A/58% B/22% C lasted for 8 min, after which it was changed in 3 min linearly to 0% A/40% B/60% C, where it was kept for 1 min to wash the column. After the wash, the elution was changed back to initial conditions for 1 min and the column was equilibrated for 6 min with initial conditions, giving a total run time of 19 min. The eluent flow rate (0.4 ml/min) was directed into the ion source of the mass spectrometer without splitting.

MS was performed using a Micromass Q-TOF1 mass spectrometer (Micromass, Altrincham, UK) equipped with an ESI Z-spray ion source. Negative ion mode ionization was used. Capillary voltage was −3000 V, sample cone voltage was −50 V, and extraction cone voltage was −2 V. The mass range acquired was *m*/*z* 200–780 with an acquisition time of 1.0 s. The desolvation temperature used was 350 °C, and the source temperature was 150 °C. Nitrogen was used as drying gas with a flow rate of 400 L/h. In MS/MS experiments, a mass resolution of 1 u