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Determination of phosphatidylethanol in blood from alcoholic males using high-performance liquid chromatography and evaporative light scattering or electrospray mass spectrometric detection

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Abstract

The 'pathologic' phospholipid, phosphatidylethanol (PEth), formed only in the presence of ethanol, was determined in extracts of human blood using high-performance liquid chromatography with evaporative light scattering detection (ELSD) or electrospray (ES) mass spectrometry. Separation was performed using a diol column and a normal-phase binary gradient system. Decreasing concentrations of PEth (15 to 1 nmol/ml blood) could be detected by ELSD in three male alcoholics, up to 3 weeks after the beginning of an alcohol-free period. Using ES, levels down to 100 pmol/ml blood was detected. The molecular species of PEth were similar to those of phosphatidylcholine found in the same blood sample. The method provides a rapid quantitative and qualitative determination of PEth in blood. The limits of detection were 200 pmol (≈125 ng) using ELSD and 140 fmol (≈100 pg) using ES, total amounts injected.

Keywords: Phosphatidylethanol; Phospholipids

1. Introduction

Phosphatidylethanol (PEth) is an aberrant phospholipid formed in cell membranes only in the presence of ethanol [1]. The formation of PEth is catalysed by the action of phospholipase D [2,3]. PEth has been found in various tissues, e.g. blood, erythrocytes, neutrophil granulocytes, brain and liver [4]. Due to the high specificity and prolonged in vivo elimination of PEth it has been suggested as a valuable marker for alcohol abuse. Blood samples are easily obtained from patients; in alcoholic patients, blood has been found to contain detectable concentrations of PEth up to 14 days after admission to an institution [5].

PEth has so far been analysed by thin-layer chromatography (TLC) which is relatively insensitive and with limited possibilities for quantitative estimation. We have therefore developed a HPLC technique with evaporative light scattering detection

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or mass spectrometry detection (MS) with electrospray ionisation (ES) for quantitative measurements of PEth in blood. The sensitivity is substantially increased compared to TLC, and the use of MS makes the analyses of the composition of PEth and other phospholipids possible.

2. Materials and methods

2.1. Chemicals and solvents

Phosphatidylethanol (PEth) standard was synthesised from synthetic phosphatidylcholine (di-18:1) by the action of phospholipase D (both purchased from Sigma–Aldrich, Sweden) [6]. The solvents used for extraction and refining of PEth from blood samples, hexane and 2-propanol (p.a. quality), were from Merck (Germany). The acetic acid (p.a.) was from Prolabo (France), triethylamine (p.a.) was from Fluka (Switzerland) and hexane, 1-propanol and water (all HPLC quality) used for HPLC analyses were from Fisons (UK).

2.2. Liquid chromatography

The HPLC system consisted of Gilson (France) 305 and 306 pumps, a Gilson 805S manometric module, a Gilson 811C dynamic mixer, a Rheodyne (CA, USA) 7125 injector, a Sedex (France) 45 evaporative light scattering detector operating at 45°C and a Shimadzu (Japan) C-R5A Chromatopac reporting integrator.

The column used was stainless steel, 250×3 mm I.D., packed with LiChrospher 100 DIOL, 5 μm (Merck, Germany) at 950 bar. The flow-rate was 0.4 ml/min and the column temperature was 55°C. A binary linear gradient was run according to the following scheme: Time 0 min (t_0)-2%B, t_{25}-100%B, t_{30}-100%B, t_{40}-2%B, t_{65}-2%B. Solvent mixture A consisted of hexane–1-propanol–acetic acid-triethylamine (86:13:0.6:0.04, v/v) and solvent mixture B of 1-propanol–water–acetic acid–triethylamine (88:11:0.6:0.04, v/v).

A standard curve of PEth using ELSD was constructed by injecting 20 μl of serially diluted PEth-standards (6–100 pmol/μl).

2.3. Mass spectrometry with electrospray ionisation

Mass spectrometry was performed with a Micromass Quattro II mass spectrometer (Micromass, Altrincham, UK) equipped with a pneumatically-assisted electrospray ionisation source. Data handling was performed with a VG Masslynx NT32 data handling system. The LC effluent entered the mass spectrometer through an electrospray capillary set at −2.4 kV (electrospray-negative mode (ES−)) at a source temperature of 120°C. Nitrogen was used both as drying gas and nebulising gas at flow-rates of 250–300 l/h and 20 l/h, respectively. Full scan spectra were obtained at a scan speed of 250 mass units/s with a mass resolution of 1 u at half peak height. A standard curve was constructed in single-ion monitoring (SIM) mode by injecting 20 μl of serially diluted PEth-standards (7–240 fmol/μl), with a mass resolution of 1 u at half peak height, a dwell time of 0.08 s and an interchannel delay of 0.02 s. All spectra were obtained using ES−. A six-port switch valve (VICI, Valco Instruments) was placed between the liquid chromatograph and the mass spectrometer. This made it possible to lead part of the sample to waste instead of into the mass spectrometer, thereby preventing unnecessary contamination of the ionisation source [7]. The sample entered the mass spectrometer only between 5 and 12 min. Between 0 and 5 min and between 12 and 65 min the sample was led to waste. Approximately 99% of the sample was wasted in this way.

2.4. Patients and controls

Three men with chronic alcoholism were recruited at the Department of Alcohol and Drug Diseases, Malmö University Hospital, Sweden. These patients had consumed 150–300 g of ethanol daily for ≥1 week prior to admission. They were followed as inpatients for about 2 weeks and thereafter as outpatients with daily health worker contacts for another 2 weeks. Two male laboratory personnel

who had abstained from alcohol for at least 4 days were used as controls.

2.5. Blood sampling and PEth extraction

A detailed description of blood sampling and extraction is given in Hansson et al. [5]. Briefly, blood sampling was performed on the morning after admission (day 1) and on subsequent mornings up to day 28 or 29. Heparinised blood (4 ml) was extracted with 150 ml of hexane–2-propanol (3:2, v/v). After filtration the solvents were evaporated and the samples re-dissolved in 500 μl of hexane–1-propanol–water (43:51:5, v/v). Mean recovery of radioactive PEth was earlier found to be 91% with a coefficient of variation (C.V.) of 32.6% estimated from 19 duplicate analyses [5]. The samples were stored at −18°C until analyses.

3. Results and discussion

PEth eluted as a well-defined peak at a retention time of 8 min using ELSD, and was baseline-separated from other compounds (Fig. 1). The compounds between 3 and 5 min were mainly triacylglycerols, cholesterol and cholesterol esters. The narrow peak at 9.2 min contained monoacylglycerols. The compounds eluting between 15 and 24 min were different phospholipid classes. Lipid classes other than PEth and monoacylglycerols were not possible to quantify in the same analysis without prior dilution. In cases where quantification of phospholipids are important, better resolution of the classes can be obtained by a slightly different solvent composition than that reported in this work [10].

The standard curve of PEth using ELSD was not linear, which is a commonly reported feature of ELSD [8,9] (Fig. 2). The correlation between dose and response was best described with a power equation (injected amount=35.526*area units$^{1.2736}$, r^2=0.997). The C.V. of the three replicates of all standard injections were <10% in all cases. The limit of detection (signal-to-noise ratio ≥5) using ELSD was 200 pmol (≈125 ng) total amount injected.

The standard curve seen in Fig. 3 using MS

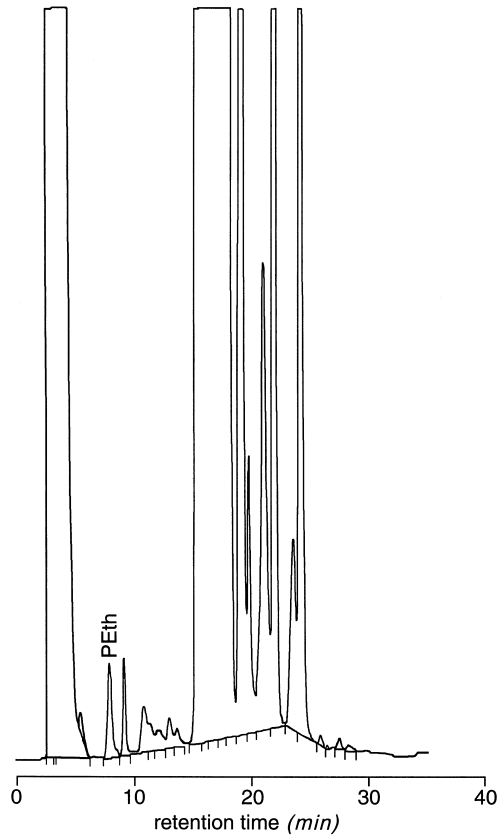

Fig. 1. HPLC–ELSD chromatogram of phosphatidylethanol (PEth) in a blood sample from a male alcoholic patient 1 day after admission. See text for information of the other compounds.

detection was constructed by analysing synthesised PEth using SIM at Da/e=727.2. The linearity over the concentration range and the limit of detection at a signal-to-noise ratio of 3 (approximately 5 pg PEth/μl) are in accordance with previous results of phospholipid analysis [10]. The C.V. of the five replicates of all standard injections were <13% in all cases. One sample (M1, day 1) was first analysed in full scan mode in order to determine the most abundant molecular ion of PEth. All samples were hereafter analysed using SIM at Da/e=701.4, the most abundant molecular ion (Fig. 4). The concentration of PEth in the samples was determined assuming that the response of each molecular species of PEth was the same as for PEth with Da/e=727.2.

The detector response of different phospholipid

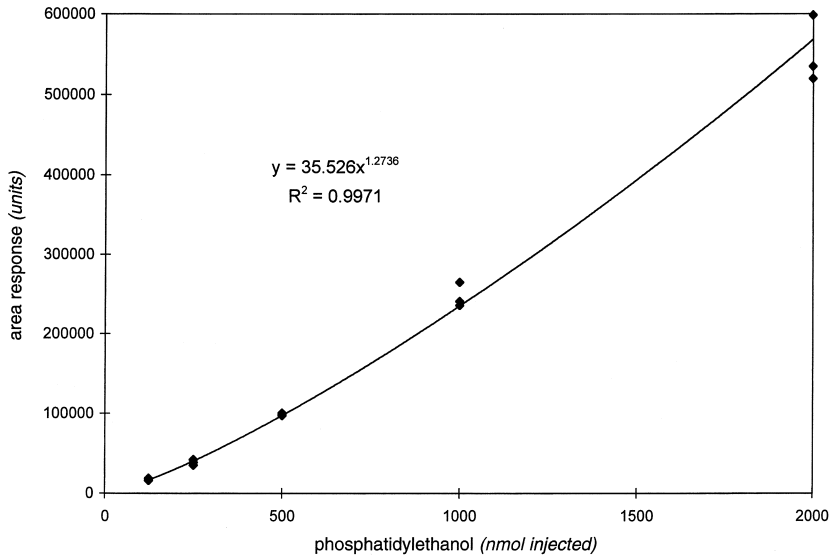

Fig. 2. Standard curve for synthetic phosphatidylethanol (PEth) using HPLC and evaporative light scattering detection.

molecular species using either light scattering or MS detection is similar for species with chains of 16 to 18 carbons. Phospholipids with a chain length of 16 to 18 carbons comprise about 90% of the total phospholipids in human blood [11,12]. Thus a reference PEth with two 18:1 chains will be a good

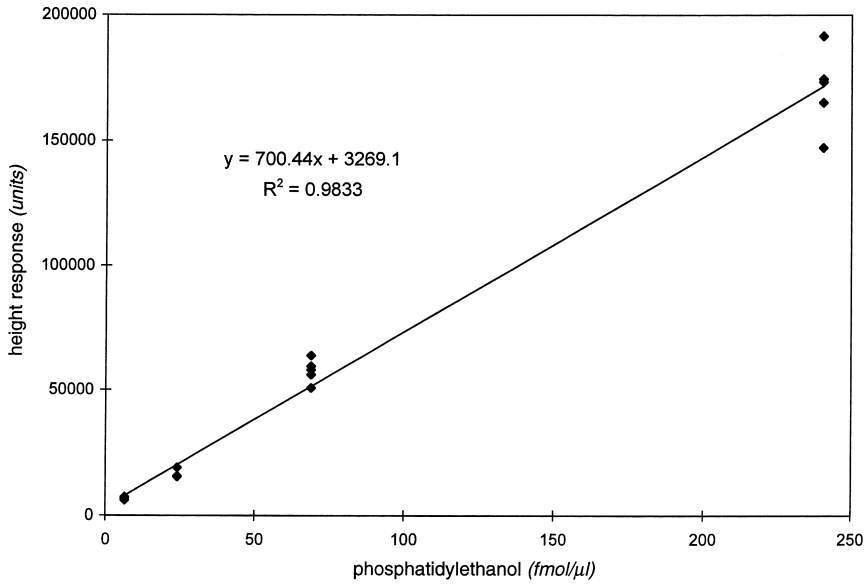

Fig. 3. Standard curve for synthetic phosphatidylethanol (PEth) using HPLC–MS(ES−) in single-ion monitoring (SIM) mode.

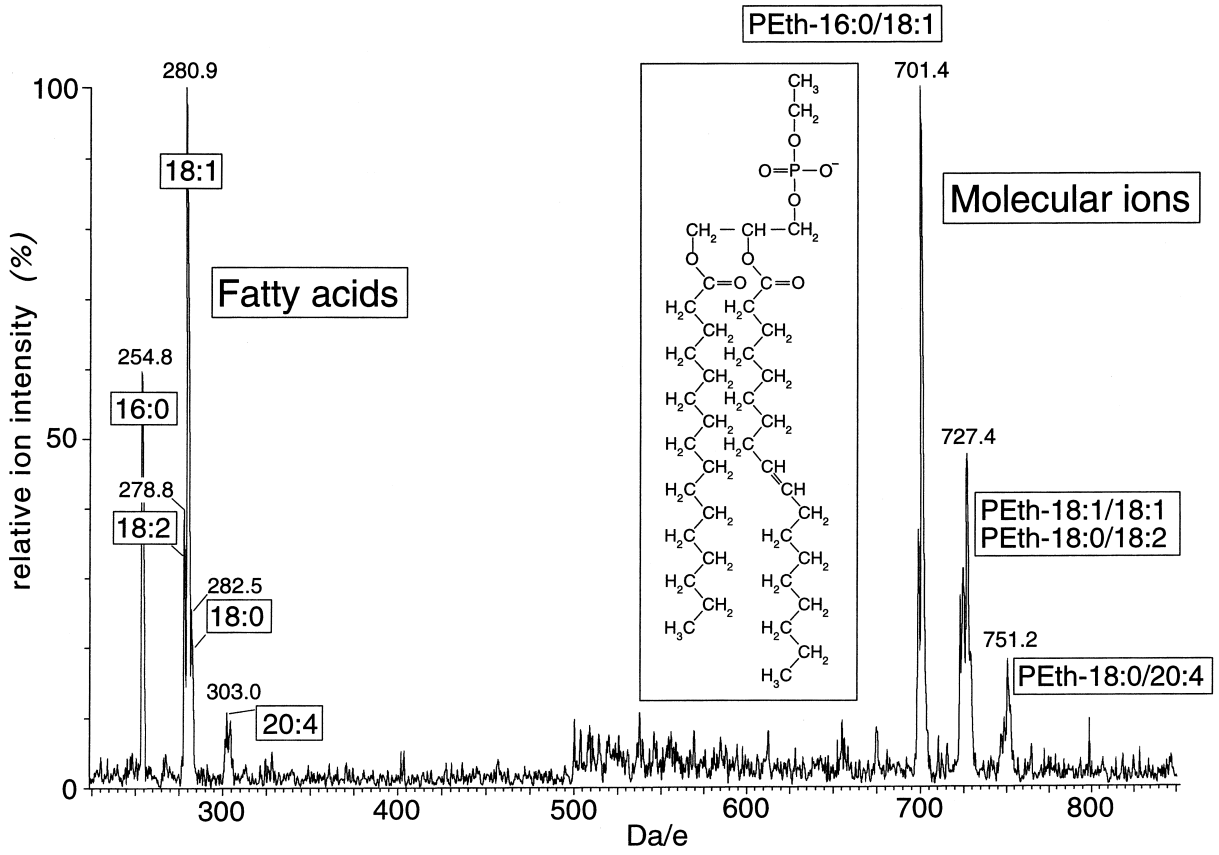

Fig. 4. Combined electrospray mass spectrum of phosphatidylethanol (PEth) in blood from a male alcoholic patient (M1) 1 day after admission. Molecular and fatty acid ions were obtained using low and high cone voltages, respectively, in one single run. Inset, structure of PEth-16:0/18:1.

representative of the mixture of PEth molecular species in the blood samples.

No PEth was detected in blood from the two male control persons [5]. The concentration of PEth in blood samples of alcoholics varied significantly between the three patients. After an initial concentration of about 5–13 nmol/ml the concentration of PEth decreased during 3–4 weeks (Fig. 5). Similar to the results found by Hansson et al. [5], PEth concentrations in some cases increased or were almost stable during the first days of withdrawal. However, to study small changes in PEth concentrations replicate samples from each day have to be analysed.

With the use of ELSD, blood PEth concentrations were detectable in patient M1 until day 14, in M2 until day 21 and in M3 still at day 29 even though the latter patient only reached a maximum concentration of 6.7 nmol/ml (at day 2). With MS (ES−) detection and SIM techniques, trace amounts of PEth, at the limit of detection, were found at day 29 (Fig. 6). The results on patient M1 obtained with MS are in good agreement with those obtained using ELSD.

The most common fatty acids of PEth were 18:1 and 16:0 (Fig. 4). 18:0 and 18:2 also occurred in significant amounts whereas 20:4 constituted less than 5% of the total fatty acids. This composition was similar to that of the most abundant phospholipid class, phosphatidylcholine (PC). A similar

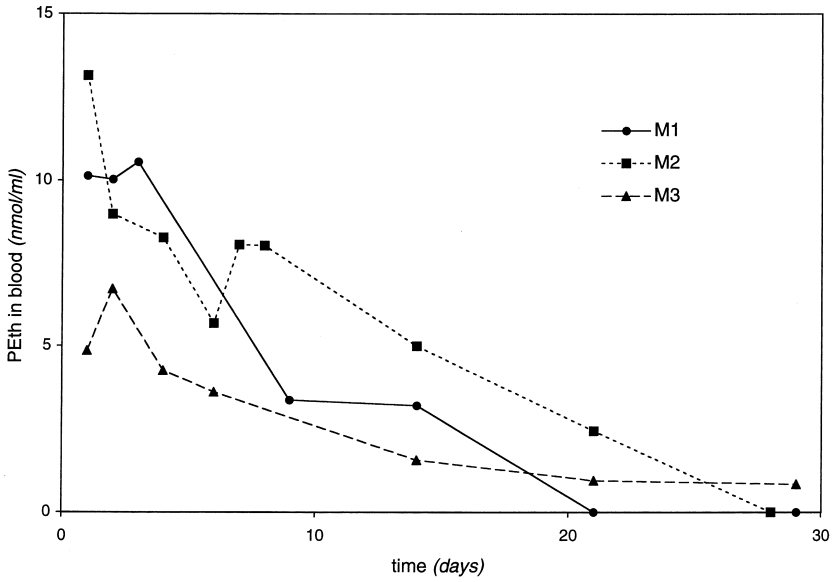

Fig. 5. Blood concentrations of phosphatidylethanol (PEth) in three male alcoholic patients (M1–M3) during 4 weeks after admission. Data were obtained using HPLC–ELSD.

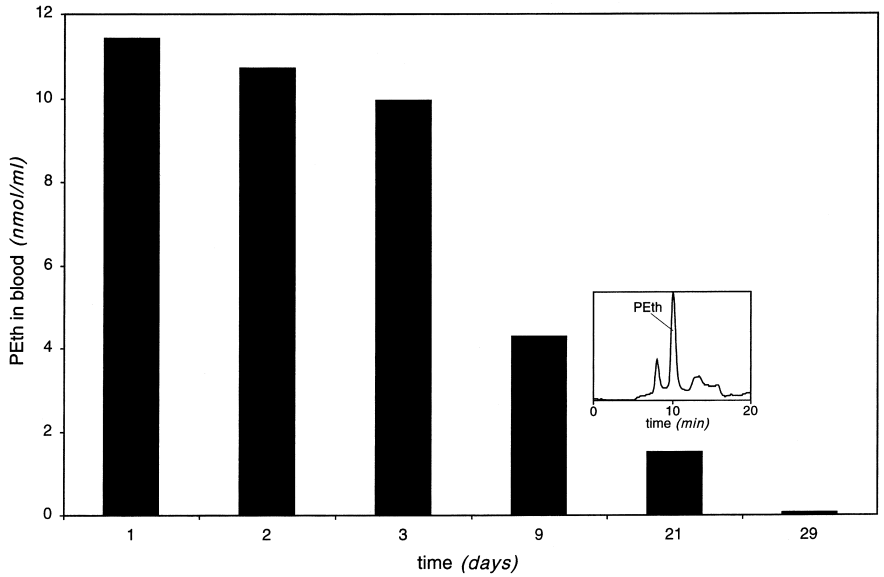

Fig. 6. Concentration of phosphatidylethanol (PEth) in blood from a male alcoholic patient (M1) determined by HPLC–MS(ES−) in single-ion monitoring (SIM) mode (Da/e=701.4). Inset, MS chromatogram in SIM mode from day 21.

molecular species composition of PEth and PC was also reported by Holbrook et al. [13].

4. Conclusion

This study demonstrates that HPLC in combination with ELSD or MS (ES−) are highly sensitive in determining PEth at low concentrations (nmol/ml blood). The set-up with HPLC–ELSD is simple and allows for easy routine analyses of blood samples from alcoholics, whereas the HPLC–MS(ES−) is preferably used when an increased sensitivity and/or knowledge about molecular species are of greater importance.

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