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BEHAVIOUR OF PLASMALOGENS DURING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ON A SILICA COLUMN WITH A MOBILE PHASE CONTAINING PHOSPHORIC ACID

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SUMMARY

Silica high-performance liquid chromatographic separation of phospho- and sphingolipids of biological origin using a mobile phase containing phosphoric acid leads to gradual hydrolysis of plasmalogens during their passage through the column. The resulting 2-acyl lyso analogues give rise to peaks that tail in the direction of the parent intact plasmalogen. Tailing can be prevented by previous complete acid hydrolysis of plasmalogens. Direct high-performance liquid chromatographic profiling of phospholipids, their plasmalogens (as 2-acyl lyso analogues) and sphingolipids is probably the method of choice for the diagnosis of patients with deficient plasmalogen biosynthesis caused by peroxisomal abnormalities.

INTRODUCTION

Phospholipids (PL) and sphingolipids (SL) are structural components of cell membranes. The fatty acid (FA) composition of each PL and SL subclass and the subclass distribution are, amongst others, determinants of the dynamic state of the membrane [1–3] and thereby of importance to its functional properties. In addition FA such as arachidonic, dihomo-γ-linolenic and eicosapentaenoic acids in PL serve as precursors of eicosanoids (prostaglandins, thromboxanes and leukotrienes) [4,5], which are potent local hormones, chemotactic and myotropic activators and modulators of platelet aggregation and inflammatory and immune reactions.

Plasmalogens differ from PL by containing an ether-linked α,β-unsaturated fatty alcohol, rather than an ester-linked FA, in the *sn*-1 position of the glycerol moiety. Plasmalogens comprise ca. 20% of all human PL [6], but their specific function is still unknown. Phosphatidylethanolamine plasmalogens (plPE) are found in all tissues and are especially abundant in erythrocytes [47% of the total

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phosphatidylethanolamine (PE)] [7,8], leukocytes and platelets [8], brain, heart and other muscles [6]. Biosynthesis of plasmalogens partly takes place in the peroxisomes [9,10]. In patients with the cerebrohepatorenal syndrome of Zellweger these peroxisomes are absent in liver and kidney, leading to a multitude of biochemical abnormalities, including deficient synthesis of plasmalogens [9–13].

PL subclasses are traditionally isolated by preparative two-dimensional thin-layer chromatography [7,14,15]. Such methods are time-consuming and therefore are gradually being replaced by isolation techniques based on high-performance liquid chromatography (HPLC) [15–20]. Using an isocratic system with a mobile phase containing phosphoric acid for the separation of erythrocyte (red blood cell, RBC) PL and SL according to Chen and Kou [16], we detected a previously unreported broad peak, between those identified as PE and phosphatidylcholine (PC), that tailed in the direction of lower retention time. We suspected this peak to originate from 2-acyl lyso-PE (2ac-lPE), which may spontaneously arise from plPE when exposed to acid. In the present study we systematically investigated the behaviour of plPE in a phosphoric acid-containing mobile phase.

EXPERIMENTAL

Materials

A ca. 1:1 mixture of bovine heart PE and plPE, soybean PE and synthetic 1-oleoyl lyso-PE (a 1-acyl lyso-PE; 1ac-lPE) were from Sigma (Brunschwig Chemie, Amsterdam, The Netherlands). HPLC-grade acetonitrile was from Baker Chemicals (Deventer, The Netherlands) and methanol from Rathburn (Brunschwig Chemie). High-performance thin-layer chromatographic (TLC) silica gel 60 plates (10 cm × 10 cm; without fluorescent indicator) and all other reagents were from Merck (Merck Nederland, Amsterdam, The Netherlands).

Isolation of erythrocytes and preparation of a total lipid extract

A 10-ml EDTA-anticoagulated blood sample from an apparently healthy adult was collected by venipuncture. The sample was centrifuged for 10 min at 800 *g*, and the plasma discarded. The RBC were subsequently washed with two 7-ml portions of 0.9% sodium chloride solution (10 min at 800 *g*) and resuspended in 0.9% sodium chloride to a haematocrit of ca. 50%.

Then 0.5 ml of the ca. 50% RBC suspension was added to 10 ml of chloroform–methanol (2:1, v/v) during vortex-mixing. The mixture was put in an ultrasonic bath for 5 min and centrifuged for 10 min at 2000 *g*. The supernatant was transferred to a tube containing 1 ml of a 5 g/l butylated hydroxytoluene (BHT) solution in methanol and evaporated to dryness at 40 °C under a stream of nitrogen. The residue was redissolved in 0.5 ml of chloroform.

Acid hydrolysis of plasmalogens

Plasmalogens in the PE + plPE standard and RBC total lipid extract were converted into their 2-acyl lyso analogues by acid treatment [7]. A 300-μl volume of a PL standard solution (containing 20 μg of PE + plPE, fortified with 500 μg

BHT) and 300 μl of the RBC total lipid extract were evaporated to dryness at 40°C under a stream of nitrogen. After addition of 100 μl of 90% acetic acid, the samples were incubated at 37°C for 18 h. Subsequently, 2.5 ml of tetrachloromethane were added. The solutions were evaporated to dryness as described above and redissolved in 300 μl of chloroform.

High-performance liquid chromatography

HPLC was performed on a Varian 5000 LC single-pump ternary solvent-delivery system (Varian Benelux, Amsterdam, The Netherlands) equipped with a Rheodyne valve injector (Inacom Instruments, Veenendaal, The Netherlands), a guard column (30 mm×4.6 mm I.D.) and an analytical column (220 mm×4.6 mm I.D.), both packed with 5-μm silica particles (Brownlee Labs., Inacom Instruments) and a Pye Unicam 4020 LC-UV detector (Philips, Eindhoven, The Netherlands) operated at an absorption wavelength of 205 nm.

Gradient elution. Mobile phases containing acetonitrile–methanol–85% (w/v) phosphoric acid in the following volume ratios were used: eluent A, 93:7:0; eluent B, 89.5:7:3.5; eluent C, 93:7:0.035. The elution programme was as follows: start, 100% C; from 0 to 1 min, linear to 100% A; from 1 to 20 min, linear to 70% A and 30% B; from 20 to 40 min, hold; from 40 to 41 min, linear to 100% B; from 41 to 45 min, hold; from 45 to 46 min, linear to 100% C. The flow-rate was 1.0 ml/min. PL fractions from the acetic acid-treated PE+plPE standard and RBC total lipid extract were collected on the basis of their retention times for further analysis by TLC (see below).

Isocratic elution. Isocratic elution was performed as described by Chen and Kou [16], using a 130:5:1.5 volume ratio of acetonitrile–methanol–85% (w/v) phosphoric acid at a flow-rate of 1.0 ml/min.

Isolation of phospholipids from fractions collected by HPLC for their analysis by TLC

To each HPLC-isolated PL fraction from the (previously acid-hydrolysed) PE+plPE standard and the RBC total lipid extract (ca. 1–3 ml) were added 2 ml of water and 3 ml of chloroform–methanol (3:1, v/v). The mixtures were vortexed and centrifuged for 5 min at 800 *g*. The aqueous (top) layers were discarded and the organic layers washed twice with 2 ml of water. The organic layers were evaporated to dryness at 40°C under a stream of nitrogen and redissolved in chloroform for analysis by TLC.

Thin-layer chromatography

TLC silica gel 60 plates were developed by a slightly modified version of the method described by Broekhuyse [14], employing the system chloroform–methanol–water–25% (w/v) ammonia (58:35:3.7:3.4, v/v). Plates were visualized by charring, according to the method described by Kaluzny et al. [21].

RESULTS AND DISCUSSION

Fig. 1 shows silica HPLC of a RBC total lipid extract without (A and C) and with (B and D) previous treatment with acetic acid and analysed by both a phos-

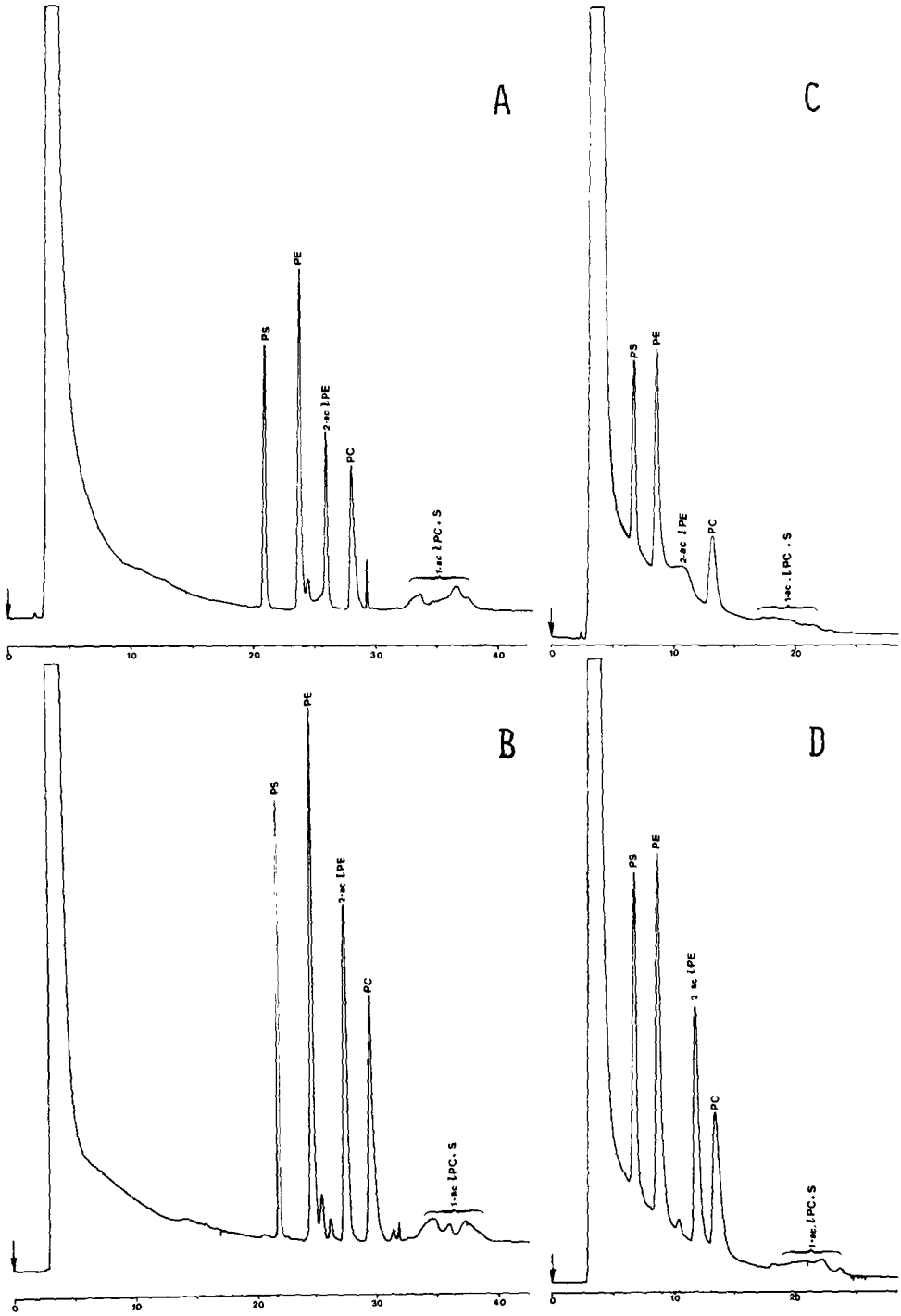

Fig. 1. High-performance liquid chromatograms of RBC phospho- and sphingolipids prepared with a phosphoric acid gradient (A and B) or an isocratic phosphoric acid eluent (C and D). Panels A and C represent chromatograms of total lipid extracts from human RBC. Panels B and D represent chromatograms from the same samples pretreated with acetic acid.

phoric acid-gradient (A and B) and an isocratic phosphoric acid eluent (C and D).

Using the isocratic system for the separation of RBC PL according to Chen and Kou [16], we observed a broad peak between PE and PC that tailed in the direction of shorter retention time (Fig. 1C). We suspected this peak, which was not reported by Chen and Kou, to originate from a plasmalogen. As outlined by these authors, the use of an acid-containing mobile phase for the analysis of complex PL and SL mixtures may lead to degradation of plasmalogens, which become converted into long-chain fatty aldehydes and their 2-acyl lyso analogues. Under less optimal separation conditions the tailing compound, tentatively identified as 2ac-lPE, may elute together with PC. In our experience this leads to abnormally high relative amounts of arachidonic acid in the PC fraction, in the case of further gas chromatographic (GC) analysis of the FA composition of the latter (for the FA compositions of the plPE and PC in human RBC see ref. 7). The tailing of the presumed 2ac-lPE could be prevented by prior treatment of the RBC total lipid extract with acetic acid (Fig. 1D).

Using a phosphoric acid gradient we obtained a much better separation of the RBC PL and SL from the solvent front (compare Fig. 1A and B with Fig. 1C and D). Direct injection of the 1:1 standard PE+plPE in this system showed two peaks, the second having the same retention time as the one tentatively identified as 2ac-lPE in the RBC total lipid extract. Tailing could be prevented by prior acetic acid hydrolysis. Acetic acid treatment had no influence on a standard PE.

Compounds giving rise to the PE peak and the tentatively identified 2ac-lPE peak of the RBC total lipid extract (Fig. 1B) and of the 1:1 standard PE+plPE were collected and further analysed by TLC. PE and plPE could not be separated with the present TLC system, but gave rise to two spots after acetic acid hydrolysis. Tentatively identified 2ac-lPE from the standard and RBC total lipid extract had similar R_F values to 1ac-lPE.

We conclude that the use of acidic mobile phases for the separation of PL and SL lipid subclasses leads to gradual hydrolysis of plasmalogens during their passage through the column, giving rise to peaks that tail in the direction of the retention times of the intact plasmalogens. The latter have shorter retention times on silica columns. Tailing can be prevented by previous complete acid hydrolysis of these PL species to their 2-acyl lyso analogues. Using such an approach in conjunction with a laser light-scattering mass detector [17,18] it should be possible to measure simultaneously the PL, SL and plasmalogen composition of biological samples on-line, which may be of importance to the diagnosis of peroxisomal disorders, like Zellwegers disease. Direct HPLC profiling of PL, their plasmalogens (as their 2-acyl lyso analogues) and SL for the determination of the relative plasmalogen content is probably faster and more sensitive than the simultaneous GC analysis of long-chain fatty aldehydes and FA methyl esters [9] and does not require previous conversion into dansyl derivatives [22].

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