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Simultaneous determination of lysophospholipids by high-performance liquid chromatography with fluorescence detection

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Abstract

A high-performance liquid chromatography (HPLC) procedure for the separation of choline lysophospholipids including 1-acyl-lysophosphatidylcholines and 1-O-alkyl-lysophosphatidyl- cholines, like the lysoform of the platelet activating factor (2-lysoPAF), is described. The lysophospholipids are derivatized at the *sn*-2 position of the hydroxyl group by 7-diethylaminocoumarin-3-carbonylazide, which converts them into the corresponding carbamoyl derivatives. The derivatized compounds were well separated by reversed-phase HPLC and quantified by fluorimetric detection. This method shows a high sensitivity and allows the separation and quantification of mixtures of lysophospholipids at picomolar level. The method was applied to assay enzyme activities, like phospholipase A_2 and PAF-acetylhydrolase, on single phospholipids or their mixtures.

Keywords: Lysophospholipids; Lysophosphatidylcholine; Platelet-activating factor

1. Introduction

Choline lysophospholipids (Cho-LPLs)[1] mainly include two classes of compounds, differing from each other with respect to the bond in the 1-position of α-glyceryl-phosphorylcholine backbone, i.e., 1-acyl-lysophosphatidylcholines (ester bond) and 1-alkyl-lysophosphatidylcholines (ether bond).

1-Alkyl-lysophosphatidylcholines are important intermediates of the ether phospholipid biochemical pathway that generates several bioactive phosphoglycerides, such as a potent autacoid lipid mediator, the 1-O-alkyl-2-*sn*-acetyl-3-glycerylphosphorylcholine (platelet activating factor, PAF) [1,2]. The unique role of PAF, which acts either as an extracellular or intracellular messenger with a wide range of biological activities, renders this mediator crucial in several physiological and pathological processes [3,4].

PAF can be synthesized by a number of cells either from dioxyacetone-phosphate (de novo pathway) or from 1-O-alkyl-2-*sn*-lyso-3-glycerylphosphorylcholine (2-lysoPAF), in turn produced either by a phospholipase A_2 (PLA_2) from 1-O-alkyl-2-*sn*-acyl-3-glycerylphosphorylcholine [5] or by a Coenzyme A independent transacylase from some acetyl-phosphoglycerides [6]. 2-LysoPAF is then

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[1] Cho-LPL: choline lysophospholipids; PAF: platelet activating factor (1-O-alkyl-2-*sn*-acetyl-glyceryl-3-phosphorylcholine); 2-lysoPAF: (1-O-alkyl-2-*sn*-lyso-glyceryl-3-phosphorylcholine); PLA_2: phospholipase A_2 (EC 3. 1. 1. 4); PAF-AH: platelet activating factor acetylhydrolase (EC 3. 1. 1. 48); DEACZ: 7-diethylaminocoumarin-3-carbonylazide.

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acetylated by an acetyl-CoA: 2-lysoPAF acetyltransferase ("remodelling" pathway) [5,6]. PAF is readily metabolized within the cells and blood plasma by a specific acetylhydrolase (PAF-AH) [7,8] that seems to have anti-inflammatory properties through the control of PAF levels [9], with generation of 2-lysoPAF. 2-LysoPAF, therefore, plays a key role in the PAF metabolism, because it is simultaneously the precursor and the catabolite of the bioactive phosphoglyceride.

1-Acyl-lysophosphatidylcholines also are bioactive lysophosphoglycerides generally produced in the plasma membrane from corresponding the phosphatidylcholines by a phospholipase A_2. 1-Acyl-lysophosphatidylcholines perform many important activities such as the induction of cell adhesion molecules [10], they effect endothelium dependent vascular relaxation [11] and perform chemotactic action on monocytes [12] or macrophages [13].

At present, the detection of Cho-LPL, sometimes present in very small amounts in biological sources, generally requires the use of radiometric techniques coupled with chromatographic techniques or methods which require expensive equipment, such as mass spectrometers [14,15], not always available in all laboratories. Some of these compounds, when possible, are detected and quantified by biological assays. For example, 2-lysoPAF can be chemically acetylated to PAF and then quantified through its aggregating property on washed rabbit platelets [16,17]. Also, the analytical determination of 1-acyllysophosphatidylcholines is hampered by somewhat cumbersome methods with inadequate sensitivity and/or specificity [18]. Therefore, the development of simple and sensitive techniques for measurement of Cho-LPL species could be very valuable in metabolic studies on these compounds.

In the present study, we developed an analytical HPLC procedure to detect, discriminate and quantify different LPL-Cho molecular species, such as 2-lysoPAF and 1-acyl-lysophosphatidylcholines, produced by enzymes that cleave the acyl moiety from choline-phospholipids. These compounds were derivatized with a highly fluorescent label at the hydroxyl group in the 2-*sn* position. Moreover, chromatographic conditions allowing the simultaneous separation and quantification of several 1-alkyl and 1-acyllysophosphatidylcholines at picomolar level were developed.

2. Experimental

2.1. Reagents and chemicals

1-O-Hexadecyl-2-*sn*-acetyl-glyceryl-3-phosphorylcholine (PAF C:16), 1-O-octadecyl-2-*sn*-acetyl-glyceryl-3-phosphorylcholine (PAF C:18), 1-O-hexadecyl-2-*sn*-lysoglyceryl-3-phosphorylcholine (2-lysoPAF C:16) and 1-O-octadecyl-2-*sn*-lysoglyceryl-3-phosphorylcholine (2-lysoPAF C:18) were from Bachem Feinchemikalien (Bubendorff, Switzerland). 1,2-Dipalmitoyl-2-*sn*-glyceryl-3-phosphorylcholine, 1,2-distearoyl-2-*sn*-glyceryl-3-phosphorylcholine, 1-palmitoyl-2-*sn*-lysoglyceryl-3-phosphorylcholine, 1-stearoyl-2-*sn*-lysoglyceryl-3-phosphorylcholine, 1-lauroyl-2-*sn*-lysoglyceryl-3-phosphorylcholine, bovine serum albumin (fraction V, fatty acid free) and snake venom PLA_2 was from Sigma (St. Louis, MO, USA). 7-Diethylaminocoumarin-3-carbonylazide (DEACZ) was purchased from Molecular Probes (Eugene, OR, USA). Anhydrous toluene, chloroform, dichloromethane, dioxane, methanol, tetrahydrofuran and choline chloride were from Fluka Chemika (Buchs, Switzerland). All other reagents were of analytical grade.

2.2. Preparation of coumarin carbamoyl derivatives of 2-lysophospholipids

The derivatization of Cho-LPL to coumarin carbamoyl derivatives was performed essentially as previously described [19]. Briefly, the samples, containing 2-lysophospholipids in 100 μl of organic phase, were dried under vacuum in Reacti-vials (Pierce) by using Univapor concentrator centrifuge Univapo 100 H (Uni Equip, Martinsried, Germany) equipment. One hundred microliters of a 1 mg/ml solution of DEACZ in anhydrous toluene (derivatizing solution) was then added into the Reacti-vials and heated at 80°C. After 3 h the vials were cooled and the content was directly analyzed by HPLC.

2.3. Separation of derivatized 2-lysophospholipids by HPLC

The separation of 2-carbamoyl coumarin derivatives of lysophospholipids was performed on a reversed-phase (RP) column Nova-Pack C_{18}, 300× 3.9 mm I.D., filled with 4 μm average particle size

(Waters, Milford, MA, USA), by a Model 32 gradient liquid chromatograph (Beckman). The elution was performed with a gradient of solvent A (methanol–water, 80:20, v/v, containing 250 mg/l choline chloride) and solvent B (chloroform). The flow-rate was 1.0 ml/min. The gradient, starting at the sample injection, was from 0 to 55% B in 22 min. The peaks were detected with a Shimadzu Model 160 fluorescence spectrometer equipped with a C-3A Shimadzu Chromatopack as integrating system (excitation 400 nm; emission 480 nm).

2.4. Enzyme assays

PAF-AH and PLA_2 activities on choline phospholipids were examined by using human serum as a source of PAF-AH and snake venom PLA_2. The incubation medium was made up by mixing 20 μl of a 1 mg/ml PAF (C:16 and C:18) in Tris-Tyrode buffer containing 0.25% bovine serum albumin and 5 m*M* EDTA, 40 μl of 0.5 mg/ml of dipalmitoyl- and/or distearoyl-phosphatidylcholine in the same buffer. The reaction was started by adding 10 μl of human normal serum. Every 10 min, aliquots of 10 μl were withdrawn and extracted with the Bligh and Dyer procedure [20]. A 20-μl volume of the organic phase was subjected to derivatization and chromatographic analysis.

As for PLA_2 action, an incubation mixture prepared as above, but in buffer containing 20 m*M* calcium chloride and no EDTA, 1.0 U/ml of snake venom PLA_2 was added. Again, for this experiment, every 10 min, aliquots of 10 μl were withdrawn and extracted with the Bligh and Dyer procedure [20]. A 20-μl aliquot of the organic phase was subjected to derivatization and chromatographic analysis.

2.5. Quantification of 2-lysophospholipids

The concentration of derivatized lysophospholipids was calculated by comparing the peak area with that of the internal standard, the DEACZ-derivatized 1-lauroyllysoglycerylphosphorylcholine.

3. Results and discussion

The purpose of the present study was to develop a sensitive method for the simultaneous chromatographic characterization and quantification of various molecular species of Cho-LPL of relevant biological interest. These lysophospholipids are different with regard to the length of their carbon chain and their type of bonding (ether or ester) at position 1 of the glyceryl moiety.

The secondary alcoholic group of the various Cho-LPLs was derivatized with the highly fluorescent reagent DEACZ which, through the very reactive isocyanate intermediate produced by heating the reaction mixture at 80°C, easily converts the 2-lysophospholipid into the fluorescent carbamoyl derivative. The reaction was practically complete within 3 h for all the compounds examined (Fig. 1), and the reaction mixture can be directly analyzed by RP-HPLC. Actually, we utilized a similar procedure to develop a non-radiochemical enzyme assay for PAF-acetylhydrolase (EC 3.1.1.7). This enzyme hydrolyzes PAF, producing acetate and 2-lysoPAF which was quantitated in this way [19].

The Cho-LPL coumarin derivatives were separated by RP-HPLC with gradient elution with a solvent composed of methanol–water (80:20, v/v) containing 250 mg/l choline chloride and chloroform as second solvent. A typical chromatogram of car-

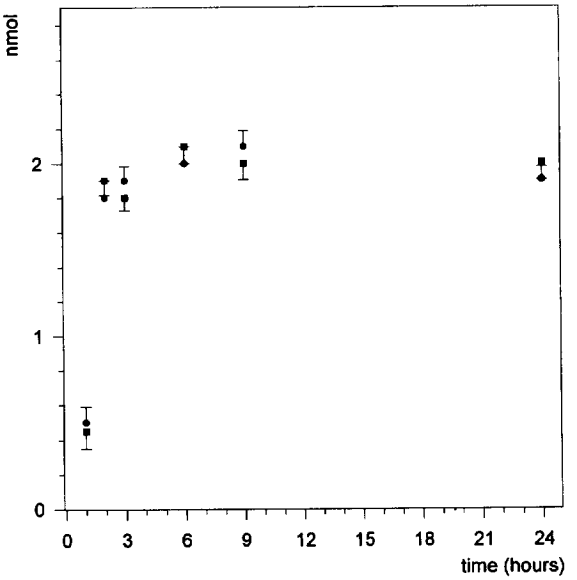

Fig. 1. Time course of the derivatization reaction of 2-lysophosphatidylcholine C:16 (●) and 2-lysoPAF C:16 (■) with DEACZ at a temperature of 80°C under the conditions reported in Section 2. The data points are the mean of three determinations.

bamoyl derivatives of an equimolar mixture of Cho-LPLs is shown in Fig. 2. It can be seen that the coumarin carbamoyl derivatives of the Cho-LPLs are well separated as sharp peaks. HPLC on a C_{18} column is able to discriminate Cho-LPLs, both on the basis of chain length and on the type of chemical bond (ether or ester) at position 1, chain length being equal. As expected, the elution time appears to be a function of chain length. Ether lipids are retained more than the corresponding ester lipids of the same chain length. The internal standard was eluted before the first peak of interest, i.e. C:16 lysophosphatidylcholine derivative. The retention times (min) of derivatized lysophospoholipids in a typical analysis were: internal standard, 11.7; lysophosphatidylcholine C:16, 14.6; 2-lysoPAF C:16, 15.1; lysophosphatidylcholine C:18, 16.1; 2-lysoPAF C:18, 16.9.

The reported chromatographic conditions gave the best resolution among many others we tried. First of all, the methanol–water ratio is crucial. A lower methanol content leads to peak broadening and does not allow high chloroform concentrations during the gradient, due to phase separation. Instead, a higher methanol to water ratio brings about a reduction of retention time and deteriorates the peak separation. Moreover, retention times that are too short are to be avoided because of the overwhelming fluorescence of the derivatizer front peaks. As far as the chloroform gradient is concerned, we observed that, under our chromatographic conditions, a faster or slower gradient than 2.5%/min of chloroform concentration increase did not improve the separation. Chloroform was also substituted with other solvents, such as tetrahydrofuran, dioxane and dichloromethane. We found that chloroform gave the best results in terms of analysis speed and resolution. However, dichloromethane, although a little less efficient than chloroform, could be a useful alternative because of its lower cost and toxicity.

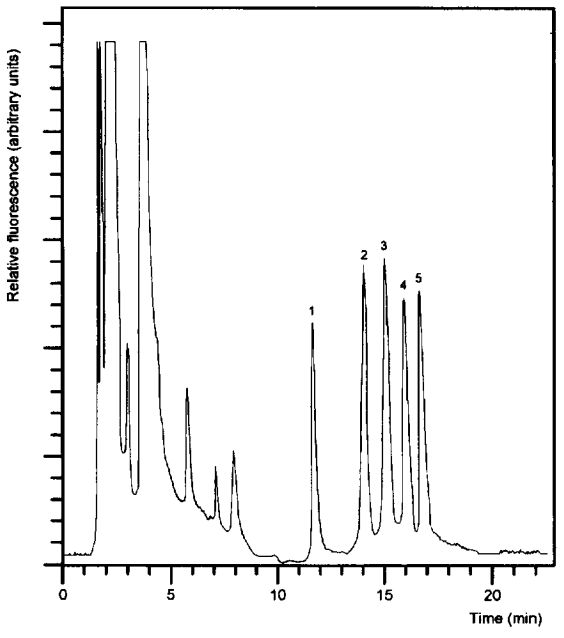

Fig. 2. HPLC of the standard mixture containing 1 mg/l of each compound. Peaks: 1=C:12 2-DEACZ-derivatized lysophosphatidylcholine, 2=C:16 2-DEACZ-derivatized lysophosphatidylcholine, 3=C:16 2-DEACZ-derivatized lysoPAF, 4=C:18 2-DEACZ-derivatized lysophosphatidylcholine, 5=C:18 2-DEACZ-derivatized lysoPAF. A 20-μl volume of the mixture was injected, as reported in Section 2.

As for the presence of choline chloride in the head solvent, it is known that choline chloride improves RP-HPLC separation of phospholipids by forming ion pairs with the polar head of these substances. Bussolino et al. [21] reported the RP-HPLC resolution of various molecular species of PAF by stepwise elution with different mixtures of methanol–water–acetonitrile and choline chloride ranging from 30 to 15 m*M*. With the eluents we used, choline chloride concentration can be utilized at a concentration substantially lower, without loss of resolution.

A linear relationship was observed between the ratios of the peak areas of the DEACZ-derivatives to that of the DEACZ-derivatized 2-lauroyllysophosphatidylcholine (internal standard) and the amount of lysophospholipids (data not shown). A calibration curve was prepared by using the carbamoyl derivative of 2-lauroyllysophosphatidylcholine. A linear relationship between the peak area and the amount of the coumarin carbamoyl derivative was obtained in the range of 5×10^{-1}–5×10^{4} pmol (data not shown). Each data point was the mean of five determinations and in each case a coefficient of variation below 5% was found.

To assess the utility of the method for biological application, the coumarin carbamoyl derivative of lysophospholipids extracted from the incubation

mixtures of phosphatidylcholines and PAF with hydrolyzing enzyme were analyzed.

In Figs. 3 and 4 two experiments of enzymatic hydrolysis of these phospholipids were reported. When a mixture of PAF C:16, PAF C:18, di-stearoyl-phosphatidylcholine and di-palmitoyl-phosphatidylcholine was incubated with human serum, extracted and derivatized, only two fluorescent peaks, corresponding to derivatized 2-lysoPAF C:16 and C:18, were seen (Fig. 3). Furthermore, these peaks increased with incubation time. Instead, when the mixture of the four phospholipids was incubated with PLA_2, four peaks with the area depending on the incubation time were produced (Fig. 4). Figs. 3 and 4 are the chromatograms from the mixtures at the end of the incubation after exhaustive hydrolysis. The results clearly show the specificity of serum PAF-AH for the ether phospholipids. Conversely, the second experiment demonstrates that snake venom PLA_2 indiscriminately cleaves ether and ester phospholipids with very different chain length at the 2-*sn* position. The carbamoyl derivatives of the various lysophospholipids were identified on the basis of comparing the retention time with those of the standard compounds.

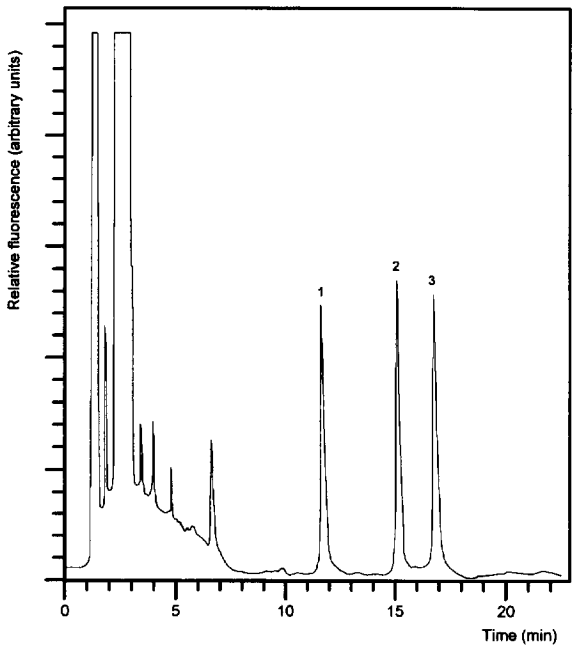

Fig. 3. HPLC of the products of the PAF-acetylhydrolase assay described in Section 2. Peaks: 1=C:12 2-DEACZ-derivatized lysophosphatidylcholine (utilized as internal standard), 2=C:16 2-DEACZ-derivatized lysoPAF, 3=C:18 2-DEACZ-derivatized lysoPAF.

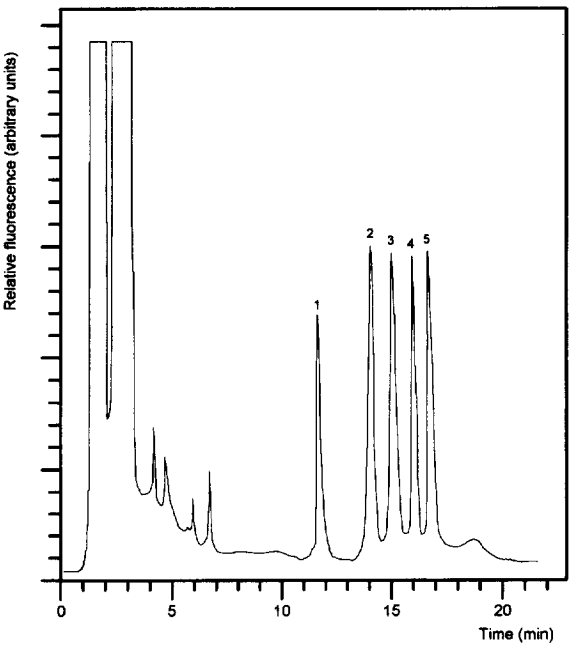

Fig. 4. HPLC of the products of the phospholipase A_2 assay described in Section 2. Peaks: 1=C:12 2-DEACZ-derivatized lysophosphatidylcholine, 2=C:16 2-DEACZ-derivatized lysophosphatidylcholine, 3=C:16 2-DEACZ-derivatized lysoPAF, 4=C:18 2-DEACZ-derivatized lysophosphatidylcholine, 5=C:18 2-DEACZ-derivatized lysoPAF.

In conclusion, the reported procedure appears to be reproducible and sensitive for quantitative analysis of two major molecular species of these classes of Cho-LPL. This approach could be useful in evaluating the participation of Cho-LPL species degrading enzymes in several patho-physiological processes. The HPLC method with fluorimetric detection using DEACZ is sufficiently sensitive for the quantitative analysis of many naturally occurring 2-lysophospholipids, such as 1-acyl-lysophosphatidylcholines and 2-lysoPAF. The high sensitivity reached could allow the reliable determination of low levels of lysophospholipids, either in natural tissues or other biological fluids. The method is simple to perform and can therefore be applied to routine analysis.

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