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Highly sensitive measurement of lipid molecular species from biological samples by fluorimetric detection coupled to high-performance liquid chromatography

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Abstract

As the molecular species composition of glycerophospholipids provides more valuable information than the corresponding fatty acid composition, we have applied a fluorimetric detection (360 and 460 nm for excitation and emission wavelengths, respectively) of anthroyl derivatives of diradylglycerol species to minor phospholipid classes and subclasses from biological samples. Diacylglycerol species were obtained by phospholipase C treatment of phosphatidylcholine subclasses and phosphatidic acid extracted from rat thymocytes. Subpicomole measurements of molecular species from the minor subclass alkenylacylglycerophosphocholine could be achieved (e.g. 0.4 pmol of the 18:1/20:5 species). Such a sensitivity allowed study of the molecular species composition of another minor phospholipid, phosphatidic acid, and to evaluation of its alteration in mitogen-stimulated thymocytes as compared to unstimulated ones. Finally, we report that such a measurement is also applicable to other minor bioactive lipids with a hydroxyl group available, namely hydroxyeicosatetraenoates (HETEs), with a similar gain of sensitivity over conventional UV detection. Overall, these measurements, especially those of phospholipid molecular species, are sensitive, reliable and meaningful for precursor–product relationship between phospholipids. © 1998 Elsevier Science B.V.

Keywords: Phosphatidic acid; Phosphatidylcholine; Phospholipids

1. Introduction

In addition to having a structural role in biological membranes, phospholipids are well recognized for their biological function, either as a store for fatty acids, especially polyunsaturated fatty acids released in response to lipase activation, or as precursors of bioactive lipids such as diacylglycerols and phosphatidates [1,2]. Instead of determining the overall fatty acid composition of glycerophospholipids by gas–liquid chromatography (GLC) of their fatty acyl methyl esters, the separation of the diradylglycerols obtained by phospholipase C treatment of those glycerophospholipids provides more relevant information on their composition in terms of the paired chains present in each molecule. In this approach, the primary alcohol of the 1,2-diradylglycerols is derivatized by a chromophore, and the derivatives may be

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separated by thin-layer chromatography (TLC) according to the radical at the sn-1 position (alkenyl, alkyl or acyl). Each subclass obtained may be then resolved into its molecular species by reversed-phase high-performance liquid chromatography (RP-HPLC), as reported in numerous papers [3–9]. The derivatization used being usually a benzoylation, the UV detection of the derivatives allows detection of 10 pmol at most, which results in difficulties in measuring molecular species from minor compounds like alkylacylglycerophosphoethanolamines, alkenylacylglycerophosphocholines as well as diradylglycerols and phosphatidates formed transiently in activated cells.

The aim of the present work was to set up a sensitive method to analyze phospholipid molecular species from biological samples as anthroyl derivatives with a fluorimetric detection on line to RP-HPLC. Examples are given for the analysis of phosphatidylcholine and phosphatidic acid in lymphoid cells with a sensitivity of 0.1 pmol, which is 100-fold higher than that obtained with classical UV detection. An attempt was also made to measure other monohydroxylated molecules such as hydroxy derivatives from arachidonic acid, i.e. HETEs.

2. Experimental

2.1. Materials

Standard phospholipids either phosphatidic acid or phosphatidylcholine (molecular species: 12:0/12:0, 14:0/14:0, 17:0/17:0, 16:0/18:2, 18:0/20:4, 18:1/18:1 and 18:2/18:2) and ricinoleic acid were provided by Sigma (St. Louis, MO, USA). 12- and 15-Hydroxy derivatives of arachidonic acid (HETE) were from Biomol (Plymouth, UK). Phospholipase C from *Bacillus cereus* was from Boehringer (Mannheim, Germany). Benzoyl chloride, thionyl chloride and 9-anthracen carboxylic acid were furnished by Sigma. Silicagel 60 plates without fluorescent indicator for TLC were from Merck (Darmstadt, Germany). All organic solvents were of HPLC quality and were from Merck or SDS (Peypin, France).

2.2. Phospholipid preparation from rat thymocytes

Lipids from $0.5 \cdot 10^9$ thymocytes, unstimulated or stimulated by 1 mg Con A/10^9 cells or 25 mg Con A/l for 5 min, were extracted according to Bligh and Dyer [10], the extracts were dried under nitrogen and submitted to bidimensional TLC, which is a crucial step to separate phosphatidic acid from other phospholipids [11]. The first eluent was chloroform–methanol–ammonia (65:35:5.5, v/v). The second eluent after thorough drying was chloroform–acetone–methanol–acetic acid–water (30:40:10:10:5, v/v). Phospholipid spots were localized by UV detection of the 2′,7′-dichlorofluorescein dye. Each phospholipid spot was scraped off and treated three times with chloroform–methanol–water (5:5:1, v/v) for extraction from the gel.

2.3. Diradylglycerol preparation from phospholipids and anthroyl derivatization

Standard phospholipids and phospholipids of biological origin were sonicated for 2 min in 1 ml of Tris–borate buffer pH 7.5 containing $3 \cdot 10^{-5}$ *M* butylated hydroxytoluene as an antioxidant. Then 50 units of phospholipase C for 200 μg phospholipids were added plus 1 ml of diethyl ether. The mixture was incubated under nitrogen overnight at 25°C with vigorous shaking. The resulting diradylglycerols were then extracted three times by diethyl ether [4].

For derivatization of the diradylglycerols obtained, 9-anthroyl chloride was first synthesized from thionyl chloride and 9-anthracene carboxylic acid according to Bayliss et al. [12]. Dried diradylglycerols were then incubated with 0.1 ml of 0.25 *M* 9-anthroyl chloride in acetonitrile for 10 min at 60°C [13]. The reaction was terminated by adding 0.5 ml of 0.1 *M* ammonia, and derivatized products were extracted three times by 2 ml of hexane and dried under nitrogen. The three diacyl, alkylacyl and alkenylacyl subclasses were separated by TLC with the eluent hexane–toluene–diethyl ether (45:50:5, v/v) [10] and localized by UV detection at R_F 0.48, 0.65 and 0.85, respectively. Each spot was scraped off and the derivatives were extracted three times by hexane–diethyl ether (1:1, v/v), the extracts were

washed with water and dried for further HPLC analyses.

2.4. HPLC separation and quantification

Molecular species from each phospholipid subclass prepared as anthroylated diradylglycerols as described above were separated by RP-HPLC using a 5 μm Hypersil ODS column (250×4.6 mm) and a flow-rate of 1.5 ml/min of the eluent acetonitrile–isopropanol (90:10, v/v). The various molecular species were identified in comparing their retention times with those of commercial standards and by GLC of their two fatty acids especially in case of unavailability of commercial standards.

Each molecular species was quantified by integrating the emitted signal (700 V except when otherwise stated) at 460 nm in response to an excitation at 360 nm [13]. Response factors were calculated over a range of molecular species includ-

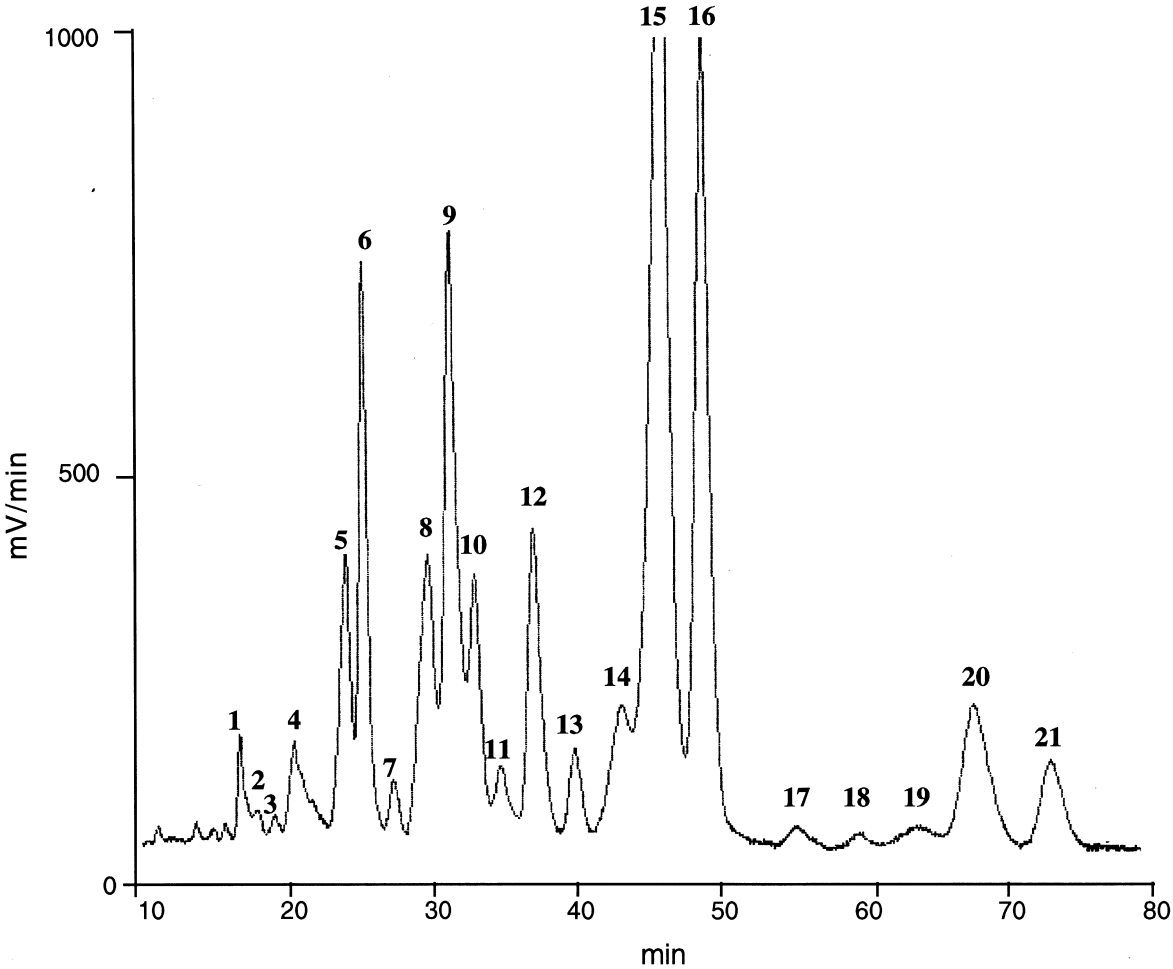

Fig. 1. Molecular species separated from diacylglycerophosphocholine. Phosphatidylcholine (PC) was isolated from rat thymocyte lipids by TLC. PC was then hydrolyzed by phospholipase C and the resulting diradylglycerols were converted into 9-anthroyl derivatives which were separated by TLC on the basis of the chemical bond at the sn-1 position (acyl, alkyl or alkenyl). The anthroyldiacylglycerol species were then separated by reversed-phase HPLC with the eluent acetonitrile–isopropanol (90:10, v/v) and detected by fluorescence at 460 nm (excitation wavelength at 360 nm). (For peak numbers, see Table 1).

ing 12:0/12:0, 14:0/14:0, 18:0/20:4 and 18:1/18:1. They did not show substantial difference and so were not considered a correction factor with the elution profiles.

2.5. Monohydroxylated fatty acids

12- and 15-HETE were first converted into methyl ester derivatives by diazomethane treatment [14], then treated with 9-anthroyl chloride to derivatize the secondary alcohol at position 12 or 15. They were analyzed by RP-HPLC with the mixture: acetonitrile–water–acetic acid–triethylamine (75:25:1:1, v/v) as the eluent [15]. Ricinoleic acid (12-OH-18:1) was derivatized the same way and used as an internal standard.

3. Results and discussion

Molecular species of phospholipid subclasses from biological samples were separated, treated with phospholipase C and derivatized as described above. In most cases, a sufficient amount of sample was separated by HPLC and molecular species were detected by UV (250 nm) for each peak to be collected and treated to obtain the two constitutive fatty acid methyl esters in order to be characterized by GLC.

As an example, Fig. 1 shows an HPLC profile of diacylglycerophosphocholine from rat thymocytes and Table 1 gives the molecular species corresponding to each peak. For each couple, it is assumed that the first fatty acid name designates the fatty acid present at the sn-1 position of the initial phospholipid. In several cases, three or four fatty acids instead of two or one in the case of di 16:0 and di 18:1 were found in one HPLC peak. This is due in some cases to a mixture of molecular species each having the same global number of carbons and double bonds. A good example is given with peak 9 with 34 carbons and two double bonds in both molecular species. In other cases the difference between the carbon numbers is exactly compensated for by the difference between the number of double bonds (e.g. peak 10 with 38:4 vs 36:3 and peak 15 with 36:2 vs 34:1). It seems grossly that two carbons equal one double bond in that matter but this is not valid for single molecular species separated as two carbon difference brought about more polarity shift than one double bond (e.g. peaks 5 and 6 and peaks 20 and 21). Two fatty acids were paired on the basis of their expected relative polarity and the literature data obtained by UV detection [3–9]. According to the fluorimetric detection sensitivity determined with known standards (see below), the amount in peaks detected varied from 1 to 45 pmol (e.g. peak 1, 2 pmol; peak 6, 12 pmol; peak 7, 1 pmol; peak 11, 1 pmol; peak 15, 45 pmol; peak 16, 29 pmol; peak 21, 4 pmol).

Table 1
Identification of RP-HPLC peaks numbered in Figs. 1–3,5

Peak No.	Molecular species
1	18:2n-6/20:4n-6; 16:1/20:4n-6
2	18:1/20:5n-3
3	18:1/22:6n-3; 16:0/20:5n-3
4	16:0/22:6n-3; 18:2n-6/18:2n-6
5	18:1/20:4n-6
6	16:0/20:4n-6
7	18:1/22:5n-6
8	18:1/18:2n-6; 18:0/22:6n-3
9	16:0/18:2n-6; 16:1/18:1
10	16:0/22:4n-6; 16:0/20:3n-6
11	18:0/22:5n-3; 16:0/20:3n-9
12	18:0/20:4n-6
13	18:0/22:5n-6
14	18:1/18:1
15	16:0/18:1; 18:0/18:2n-6
16	16:0/16:0
17	18:0/20:3n-9
18	18:0/20:2n-6
19	18:1/20:1n-9
20	18:0/18:1; 16:0/20:1n-9
21	16:0/18:0
22	18:0/22:4

Peaks were identified by comparison with standards or by GLC determination of their fatty acid content.

After separation of the alkylacyl and alkenylacyl subclasses from ether phospholipids, as described in Section 2.1, peak numbers were attributed according to Table 1, although the GLC analysis of the molecular species for their characterization did not provide any information on the sn-1 position of alkylacyl derivatives as the transmethylation procedure does not work on alkyl groups. HPLC peaks were then assigned according to the retention time shifts compared to diacyl groups and according to literature data available from UV analyses [3–9]. For

alkenylacyl derivatives, HPLC peaks were identified according to the dimethylacetal GLC analysis corresponding to the sn-1 position. Examples of HPLC analysis of alkylacylglycerophosphocholine and alkenylacylglycerophosphocholine from rat thymocytes are given in Figs. 2 and 3, respectively. It can be noticed first that ''corresponding'' peaks (irrespective to the acyl, alkenyl or alkyl nature of the sn-1 position) show retention time shifts toward higher values for alkenylacyl and even higher for alkylacyl derivatives, in agreement with their respective lower polarities. Second, it is worthwhile to note that even minor components as alkenylacylglycerophosphocholine could be analyzed with the present method (Fig. 3) with peaks amounting from 0.4 pmol (peak 2) to 4.7 pmol (peak 12). The major molecular species of this phospholipid subclass (peak 22, 6 pmol) was characterized as 18:0/22:4.

The sensitivity for the detection of those molecular species allowed subpicomole measurements. As a matter of fact we made some standardization with dilauroylglycerol, keeping the signal-to-noise ratio above 5. A good linear response could be obtained between 0.1 and 1 pmol of standard (correlation coefficient of 0.996 for five experimental points). Such a sensitivity is in the range of 10 to 100-fold higher than that available with the UV detection of similar chromophores. The sensitivity appears even greater than that anticipated for 1-anthroyl derivatives [16] and of course much higher than that for methods aiming to separate phospholipid species keeping their polar head [17]. We found the tech-

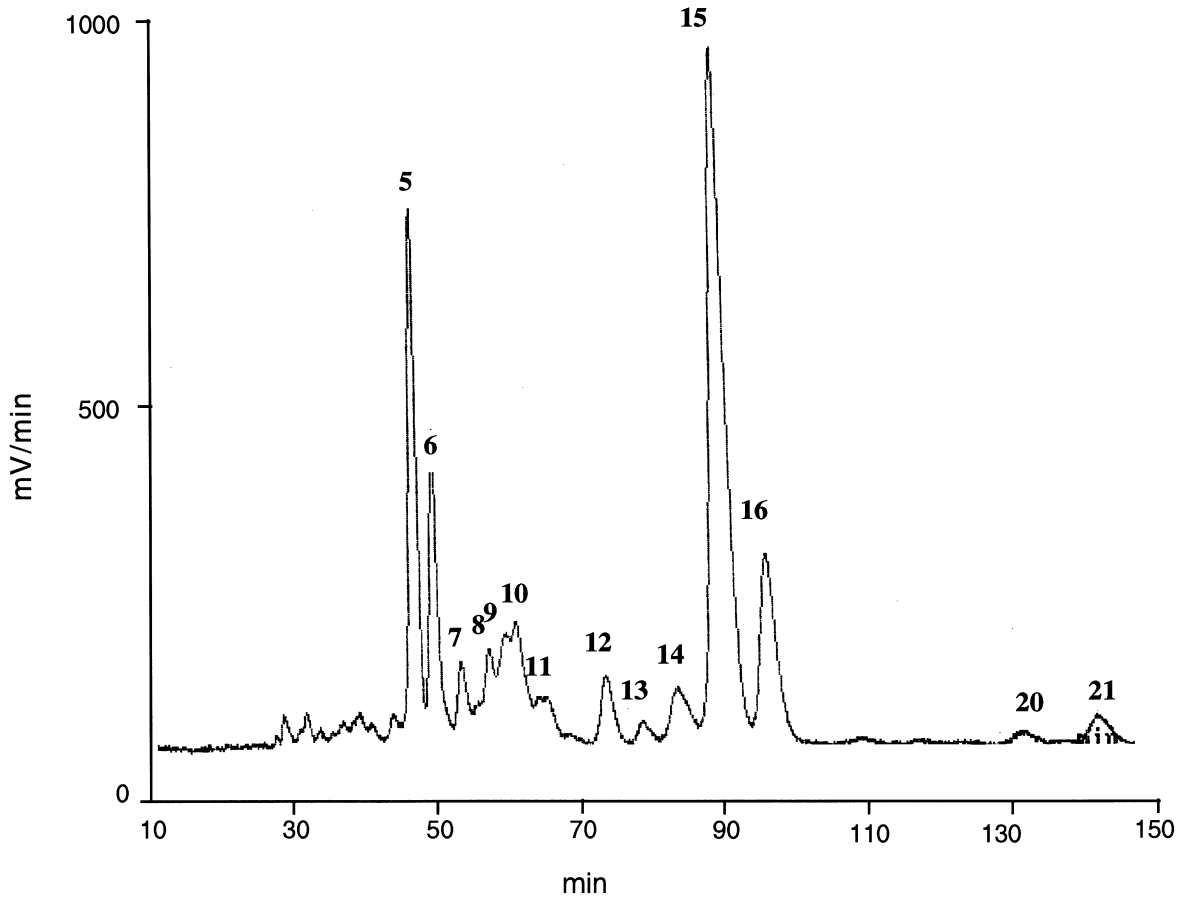

Fig. 2. Molecular species separated from alkylacylglycerophosphocholine. The anthroylalkylacylglycerol species were obtained and analyzed as described for diacylglycerol species (see legend to Fig. 1).

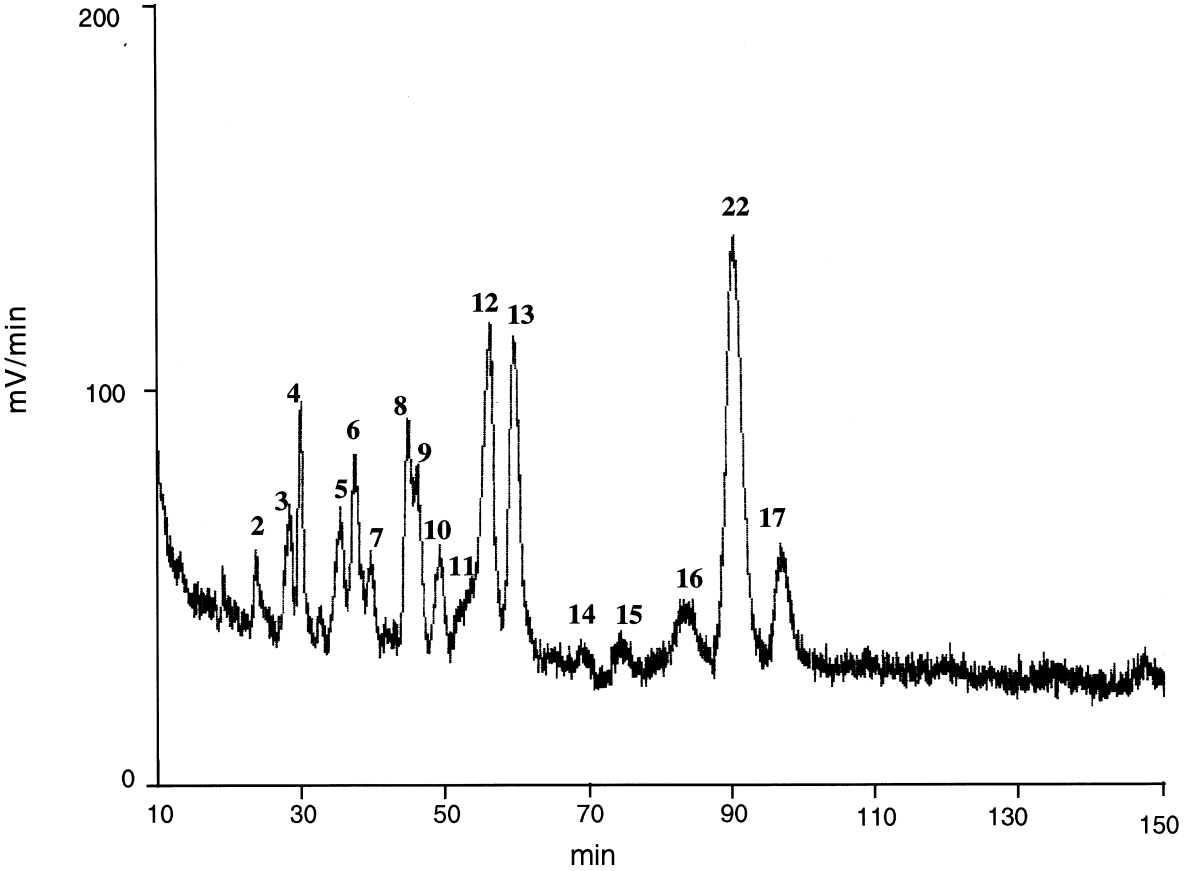

Fig. 3. Molecular species separated from alkenylacylglycerophosphocholine. The anthroylalkenylacylglycerol species were obtained and analyzed as described for diacylglycerol species (see legend to Fig. 1).

nique applicable to other hydroxy bioactive compounds like monohydroxy derivatives of arachidonic acid, namely HETEs, with the same gain over the UV detection of underivatized HETEs sensitivity (Fig. 4). In this case, the HETEs were first transformed into methyl esters by diazomethane treatment, converted into 9-anthroyl derivatives and separated as described in the Section 2. It is however noteworthy that the hydroxyl group derivatization prevented the separation of the positional isomers as derivatized 12-HETE and 15-HETE comigrated (Fig. 4), in contrast to what was observed with the underivatized compounds. Nevertheless, this might be considered as an advantage in experiments aiming to measure total HETEs as a reflection of the overall arachidonic acid hydroxylation. The sensitivity attained was quite similar to that obtained with anthroyldilauroylglycerol, i.e., a linear detection from 0.1 pmol.

We next focused our attention on phosphatidic acid, which is produced in small amounts in stimulated cells [18] where it could play a role as a lipid mediator [19,20]. At least ten major molecular species could be detected and measured in the picomole range in unstimulated cells. As expected the profile of phosphatidic acid from resting thymocytes was close to that of diacylglycerophosphocholine, except for species containing arachidonic acid (20:4), which were slightly more abundant in phosphatidic acid (around 10% for each 16:0/20:4 and 18:0/20:4 in phosphatidic acid versus 6% in diacylglycerophosphocholine). The difference

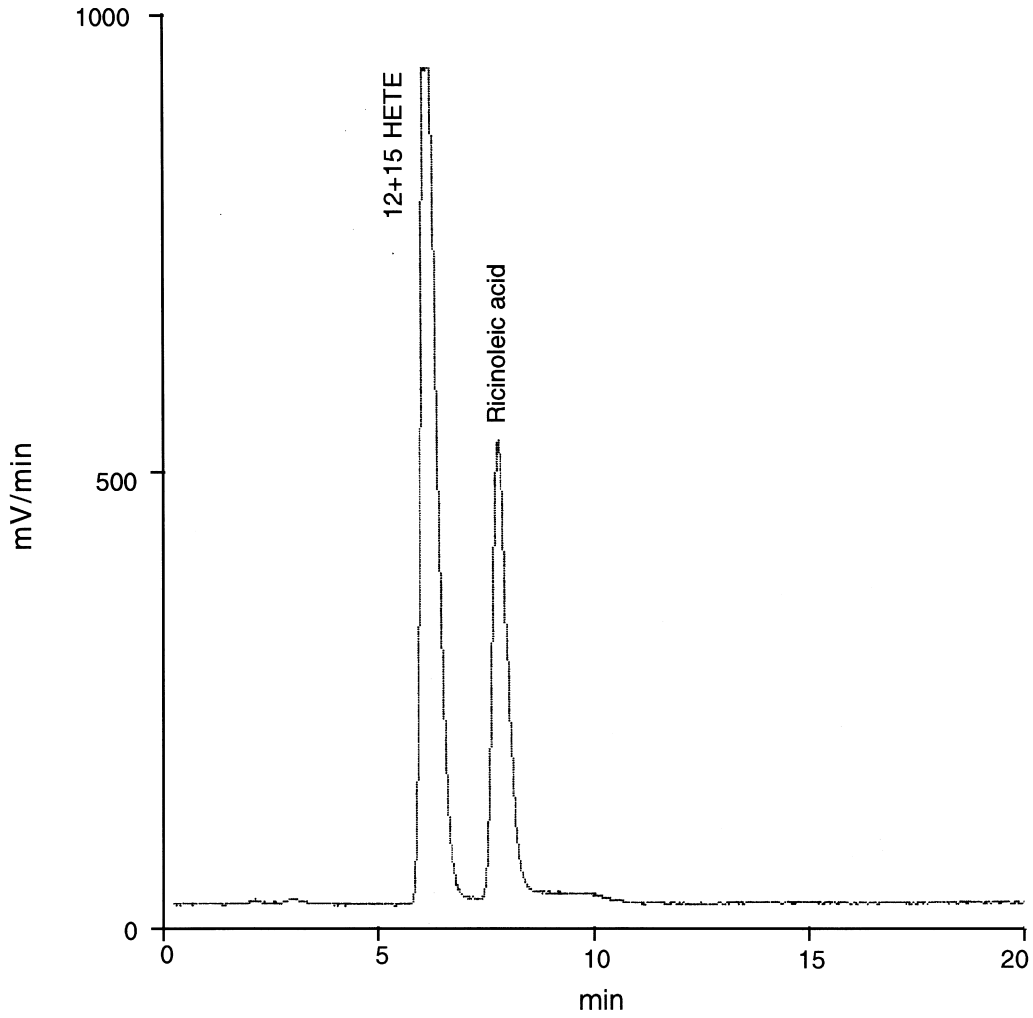

Fig. 4. Separation and detection of anthroyl derivatives of hydroxylated fatty acid methyl esters. The hydroxylated fatty acids were 12- and 15-HETE, the end-products of arachidonic acid via the 12- and 15-lipoxygenases, and ricinoleic acid. The reversed-phase HPLC was done with the eluent acetonitrile–water–acetic acid–triethylamine (75:25:1:1, v/v) with the same detection as in Fig. 1. A 4 pmol quantity of each hydroxylated fatty acid was injected.

was much higher in phosphatidic acid from thymocytes stimulated by the mitogen Con A where the proportion of 18:0/20:4 species attained 27% of the total (Fig. 5). This example illustrates how the methodology described here may allow the determination of which phospholipid molecular species may be specifically produced in response to cell activation. Comparing the molecular species between several phospholipid classes may then give information about the possible precursor–product relationships. In the present example, the higher richness of phosphatidic acid in 16:0/20:4 and 18:0/20:4 compared to diacylglycerophosphocholine from the same cell population makes it unlikely that the former derives massively from the latter. The arising of the 18:0/20:4 species in response to mitogen activation emphasizes this and suggests rather that diacylglycerophosphoinositol is likely to be the main source for the newly produced phosphatidic acid [21].

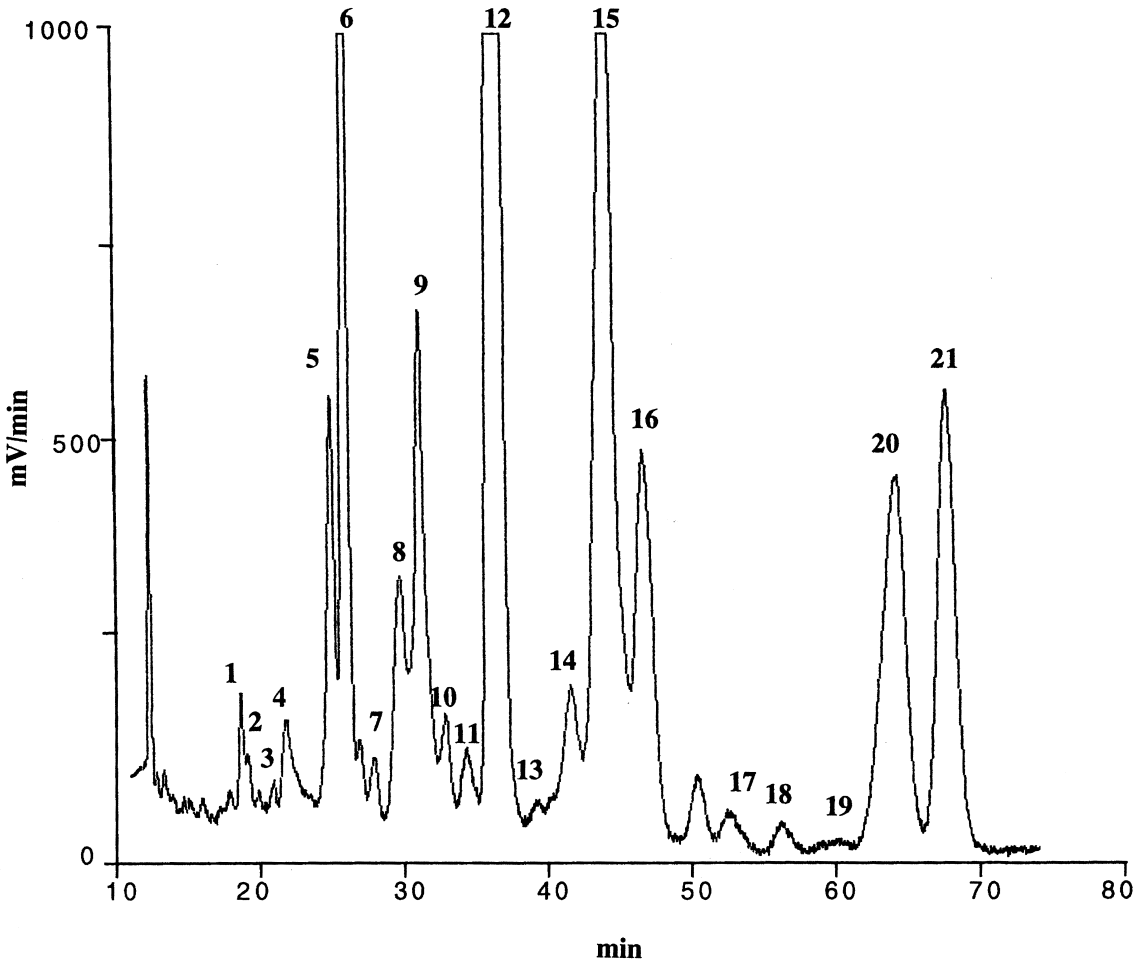

Fig. 5. Molecular species separated from phosphatidic acid purified from Con A-stimulated rat thymocytes. Phosphatidic acid was purified by two-dimensional TLC and treated as phosphatidylcholine (PC) for measuring the diacylglycerol species (see legend to Fig. 1). The major molecular species of the profile derives from stearoyl,arachidonoylglycerol phosphate (peak 12).

We conclude from these studies that the HPLC separation of diradylglycerol moieties derived from glycerophospholipids followed by fluorimetric detection is a very sensitive method applicable to minor phospholipid classes from biological samples. Such a fluorimetric detection has also been successfully applied to monohydroxylated fatty acids.

Acknowledgements

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