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SENSITIVE METHOD FOR THE ANALYSIS OF PHOSPHOLIPID SUBCLASSES AND MOLECULAR SPECIES AS 1-ANTHROYL DERIVATIVES OF THEIR DIGLYCERIDES

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SUMMARY

A sensitive high-performance liquid chromatographic (HPLC) method for the separation and quantitation of phospholipid subclasses and molecular species has been developed. Phospholipids for analysis are hydrolyzed to the diradyl glycerols (DGs) with phospholipase C and the resulting DGs reacted with a molar excess of 1-anthroyl nitrile in the presence of quinuclidine or 4-dimethylaminopyridine to form a stable adduct. The anthroyl-DGs were separated into alkenylacyl, alkylacyl, and diacyl subclasses either by using normal-phase HPLC or by thin-layer chromatography on silica gel G plates. Molecular species within alkenylacyl, alkylacyl, and diacyl subclasses were separated using reversed-phase HPLC. Separation of the individual subclasses was achieved for ethanolamine phosphoglycerides from bovine brain, as well as choline and ethanolamine phosphoglycerides from human neutrophils. Separation and quantitation of individual molecular species were carried out for alkenylacyl, alkylacyl, and diacyl subclasses of bovine brain ethanolamine phosphoglycerides by their absorbance at 254 nm with correction for recoveries as normalized to the internal standard 1,2-dipentadecanoyl-3-phosphatidylcholine added before the hydrolysis of phospholipids with phospholipase C or 1,2-dipentadecanoyl-3-anthroyl glycerol added after com-

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plete derivatization. The extinction coefficient of the 1-anthroyl derivatives were greater than 68 000 permitting the generation of concentration-dependent determinations which were linear to less than 1 pmol when monitored at 254 nm. Thus, this procedure provides a new and very sensitive method for the quantitation of picomole quantities of phospholipids or DGs by HPLC techniques.

INTRODUCTION

The structural elucidation of the platelet activating factor (PAF) as a phospholipid [1], and subsequent suggestions that PAF and the eicosanoids are derived from a common intermediate [2–4], has renewed interest in the detailed analysis of phospholipids. Traditionally, the analysis of phospholipid subclasses and their molecular species has been a formidable task. The nonvolatility of these compounds has precluded analysis by gas chromatography (GC) or gas chromatography–mass spectrometry (GC–MS), and their ionic nature has restricted chromatography on normal-phase systems [5,6]. Although reversed-phase high-performance liquid chromatographic (HPLC) methods (with ion suppression) are now available for separation of phospholipid molecular species [7,8], the peak shape is poor, the analyses are long, and detection often requires microgram quantities.

These difficulties have prompted many investigators to look for phospholipid derivatives which allow opportunities for greater chromatographic resolution and which have higher molar extinction coefficients. This has been accomplished by chemical modification of the intact phospholipid [9,10] or by enzymatic hydrolysis of the phosphobase and subsequent derivatization of the diglycerides [11–15]. Except for lipid derivatives with a chromophoric group [11,13–15], identification and quantitation of the separated species required additional steps such as collection of the individual components separated by HPLC and methylation of constituent fatty acids followed by GC analysis or a direct identification and quantitation of GC or HPLC separated components by MS. For lipid derivatives with chromophoric groups [11,13,15], detection often requires nanogram quantities of material because of the modest extinction coefficients of the chromophores. This could be a limiting factor in cases where the mass available for analysis is quite low (as the case of cellular PAF and diglycerides) and where the intent is to quantitate small changes in the phospholipid molecular species in response to specific stimulation.

We have recently developed a very sensitive assay for PAF and lyso-PAF analyses [16]. After cleaving the phosphocholine with phospholipase C, the resulting diradyl glycerol (DG) was converted to the pentafluorobenzyl (PFB) ester which generated a high yield of stable negative ions when analyzed by MS under chemical ionization conditions permitting detection and quantitation of femtomole quantities of the material.

Although the PFB derivatives were very suitable for short-chain DGs, the

derivatization of long-chain DGs to PFB esters was often incomplete and these derivatives were not volatile enough to be analyzed by GC or GC–MS. In order to overcome this problem and to increase the sensitivity of detection, 1-anthroyl nitrile was chosen as a new reagent for the derivatization of long- or short-chain DGs for analysis by HPLC. Because of the high extinction coefficient of the anthroyl group, the limit of detection is much lower and the sensitivity much better than currently used chromophores such as benzoates [13] or dinitrobenzoates [15]. Therefore, the present method provides a useful tool needed for the analysis of small amounts of phospholipids and diacylglycerols. In the present work, we have applied this method for the separation and quantitation of subclasses from bovine brain ethanolamine phospholipids and human neutrophils choline and ethanolamine phospholipids. In addition, the separation and quantitation of individual molecular species from alkenylacyl, alkylacyl, and diacyl subclasses of bovine brain ethanolamine phospholipids have been achieved.

EXPERIMENTAL

Materials

1-Anthroyl nitrile was prepared from anthracene-1-carboxylic acid as described [17]. The crystalline material was desiccated and stored at −20°C for over a year without degradation. 4,4-Dimethylaminopyridine (DMAP), molecular sieves, PFB bromide and quinuclidine were purchased from Aldrich (Milwaukee, WI, U.S.A.). Ethanolamine phospholipids from bovine brain, diisopropylethylamine, phospholipase C from *Bacillus cereus*, butylated hydroxytoluene (BHT) and primulin dye were from Sigma (St. Louis, MO, U.S.A.). 1-Palmitoyl- and 1-stearoyl-*sn*-glycerol-3-phosphocholines, containing palmitic, stearic, oleic, linoleic, and arachidonic acids in the sn_2 position, 1-O-hexadecyl-2-hydroxy-*sn*-glycerol-3-phosphocholine (lyso-PAF) and dipentadecanoyl-*sn*-glycerol-3-phosphocholine were purchased from Avanti Polar Lipids (Birmingham, AL, U.S.A.). 1,2-Dipentadecanoyl, 1,2-dipalmitoyl, and 1,2-distearoyl glycerols, monoarachidonyl glycerol and fatty acid anhydrides were purchased from Nuchek (Elsian, MN, U.S.A.). 1-O-Hexadecyl-*sn*-glycerol-3-phosphocholine species containing palmitic, stearic, oleic, linoleic, and arachidonic acids in the sn_2 position , were synthesized using lyso-PAF and the corresponding fatty acid anhydrides [18]. 1-Palmitoyl-2-[1-^{14}C]oleoyl-*sn*-glycerol-3-phosphocholine (specific activity 55 mCi/mmol) and 1-palmitoyl-2-[1-^{14}C]arachidonoyl-*sn*-glycerol-3-phosphocholine (specific activity 54.5 mCi/mmol) were purchased from Dupont-NEN Radiochemicals (Boston, MA, U.S.A.).

All solvents used were of HPLC grade and were obtained from Burdick and Jackson (Muskegon, MI, U.S.A.). Acetonitrile used in derivatization was made

anhydrous by storing over molecular sieve 4A (8–12 mesh). All test tubes were siliconized before use.

Isolation of phospholipids from neutrophils

Venous blood from healthy donors was collected in ethylene glycol tetra-acetate (EGTA) containing tubes and the neutrophilic polymorphonuclear leukocytes (PMNLs) were isolated using Monopoly Resolving Media (Flow Labs, McLean, VA, U.S.A.) [19]. Lipids from PMNLs were extracted by the procedure of Bligh and Dyer [20]. The PMNL lipids were dissolved in 100–200 μl of chloroform–methanol (2:1, v/v), spotted on 500 μm thick silica gel G thin-layer plates (AnalTech, Newark, DE, U.S.A.), and choline- and ethanolamine-containing phospholipids were separated by using chloroform–methanol–water (65:25:4.5, v/v) as solvent. The individual phospholipid bands were made visible by spraying the thin-layer chromatography (TLC) plates with 0.01% primulin dye in methanol and viewing under ultraviolet (UV) light; the phospholipids were eluted from silica gel by the procedure of Bligh and Dyer [20] and reconstituted in toluene–ethanol (2:1, v/v) containing 0.01% BHT.

Preparation of diglycerides

Solutions of synthetic phospholipids (10–50 μg) or phospholipids isolated from PMNLs (from $2 \cdot 10^6$ to $4 \cdot 10^6$ cells) or bovine brain phospholipids (10–50 μg) were dissolved in 200–400 μl of chloroform–methanol (2:1, v/v) and added to screw-capped test tubes. After the solvent was evaporated under a stream of nitrogen, 1 ml of diethyl ether and 0.5 ml of sodium borate buffer (0.05 mol/l, pH 8.0) containing 1.0 mol/l calcium chloride and 0.01 mol/l zinc chloride were added, and this was sonicated in a sonic water bath (Bronson) for 1 min. Into each test tube 25–40 U of phospholipase C enzyme from *B. cereus* were added, and the tubes were capped and vigorously shaken at 37°C for 2 h in a water bath [16]. The diethyl ether was evaporated under a stream of nitrogen and the DGs were extracted three times with 2 ml of diethyl ether. The ether extracts were pooled and evaporated under a stream of nitrogen to absolute dryness and then rapidly derivatized to 1-anthroyl ethers.

Preparation of diglyceride-anthroyl ethers

Synthetic DGs or DGs derived from phospholipids were dissolved in 100 μl of anhydrous acetonitrile in each tube. Anhydrous acetonitrile (25–50 μl) containing either 5 μl of diisopropylethylamine, 2–5 μg of quinuclidine, or 2–5 μg of DMAP was added followed by 100 μl of a 1 μg/μl solution of 1-anthroyl nitrile in anhydrous acetonitrile. After flushing with nitrogen, each tube was capped and incubated for desired periods of time at 70°C. The reaction was terminated by adding 100 μl of methanol, the solvents were evaporated with nitrogen, and the residue was either reconstituted in cyclohexane–diethyl ether

(90:10, v/v) and analyzed by normal-phase HPLC or reconstituted in chloroform–methanol (9:1, v/v) and analyzed by TLC. The derivatives were stable for several months at 0–4 °C.

TLC and HPLC procedure

The extent of derivatization of DGs to DG-3-[1-anthroyl] ethers was monitored by spotting a 10–25 μl aliquot of the reaction mixture on a silica gel G plate and developing the TLC plate using hexane–diethyl ether (70:30, v/v). In this system the 1-anthroyl ethers separated as a broad band (R_F from 0.8 to 0.92) whereas the unreacted DGs had an R_F of 0.22.

Separation of DG-anthroyl derivatives into alkenylacyl, alkylacyl and diacyl subclasses was achieved either by TLC or HPLC.

1,2-Diradyl-3-[1-anthroyl]-glycerols were spotted on silica gel G plates and developed using toluene–hexane–diethyl ether (10:8:0.3, v/v). The plates were dried under a stream of nitrogen and viewed under UV light. With lipids derived from biological samples, three well separated intense fluorescent bands were visible with R_F values of 0.52, 0.67, and 0.80 which represented diacyl, alkylacyl, and alkenylacyl subclasses, respectively, as determined by appropriate standards. The subclasses were extracted from the silica using chloroform and reconstituted in acetonitrile–isopropanol (70:30, v/v).

Separation of these subclasses was also achieved using a Shimadzu HPLC system with a variable-wavelength detector and a Model C-R4A data processor. A 45 mm$\times$4.6 mm precolumn packed with 10-μm Ultrasphere silica and a 250 mm$\times$4.6 mm analytical column packed with 5-μm Ultrasphere silica were used for separation of the subclasses. Samples were injected in 10–25 μl of the mobile phase and eluted isocratically using cyclohexane–diethyl ether (98:2, v/v) at a flow-rate of 2 ml/min, and the absorbance was monitored at 254 nm.

Separation of molecular species within each subclass of ethanolamine phospholipid from bovine brain was accomplished using the above HPLC system except that precolumn and analytical column were packed with 10- and 5-μm Ultrasphere ODS (C_{18}), respectively, acetonitrile–isopropanol (70:30) was used as the mobile phase at a flow-rate of 3 ml/min, and 1-ml samples were collected.

Gas chromatography

The individual subclasses of bovine brain ethanolamine phospholipids were separated by either TLC or normal-phase HPLC as described above. The major molecular species from the individual subclasses were separated by reversed-phase HPLC and the fatty acid composition was determined by GC. To each collected fraction 200–500 ng of monoarachidonyl glycerol were added and the solvent evaporated to dryness under nitrogen. The samples were then saponified by the addition of 500 μl of 0.3 *M* methanolic potassium hydroxide

and incubation for 30 min at 37°C. After acidifying to pH 2 with 2 *M* hydrochloric acid, the free fatty acids were extracted by ethyl acetate and derivatized to PFB esters [21]. Analysis of the fatty acid PFB esters was performed on a Shimadzu Model 15A gas chromatograph equipped with an electron-capture detector, AOC-6 autoinjector and C-R4A data processor. The PFB esters were separated on a fused-silica capillary column (15 m×0.25 mm I.D.) coated with SP2330 phase (Supelco, Bellafonte, PA, U.S.A.). Injector and detector temperatures were maintained at 260 and 290°C, respectively. Initial column temperature was 60°C which was increased to 160°C at a rate of 30°C/min and then to 240°C at a rate of 10°C/min. Helium was used as the carrier gas (0.8 ml/min) and nitrogen was used as makeup gas. Aliquots of 2 μl sample were injected in the splitless mode and the PFB esters were identified using appropriate standards and quantitated using the internal standard eicosanoic acid which had C value of 20.

RESULTS AND DISCUSSION

Optimal conditions for each step in the isolation and derivatization sequence were worked out using a mixture of radiolabeled and unlabeled phospholipids from human PMNLs or bovine brain. The hydrolysis of phospholipids to DGs was always >98% (99±0.7%, $n=6$), based on the recovery of the radioactivity in the DG fraction and by the absence of any detectable bands corresponding to either intact phospholipids or their lyso derivatives. The rate of derivatization of DG with 1-anthroyl nitrile was significantly influenced by the type of organic base used as catalyst (Fig. 1); with either quinuclidine or DMAP the reaction was almost complete within 30 min at 70°C, by which time >95% (96.3±1.2%, $n=6$) of the radioactivity was present as DG-3-[1-anthroyl] derivative (R_F 0.8–0.92) as judged by TLC using hexane–diethyl ether (70:30, v/v) as solvent. There was also no indication of the presence of any unreacted

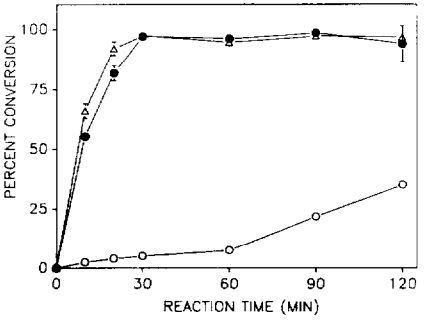

Fig. 1. Influence of organic bases on the time course for derivatization of diradyl glycerol with 1-anthroyl nitrile. (○) Diisopropylethylamine ($n=2$); (●) quinuclidine ($n=3$); (△) DMAP ($n=5$).

unlabelled DGs (R_F 0.22) on TLC. On the other hand the derivatization with diisopropylethylamine was much slower (Fig. 1). Therefore, DMAP was used routinely as the organic base and the reactions were terminated after 30 min at 70°C. The presence of even a trace amount of moisture or alcohol strongly inhibited the reaction, as was indicated by a decrease in the intensity of the yellow color of the reaction mixture.

In some experiments a known quantity of 1,2-dipentadecanoyl-*sn*-glycerol-3-phosphocholine (50 nmol) was mixed with various amounts of 1-O-hexadecyl-2-arachidonoyl-*sn*-glycerol-3-phosphocholine (10–500 nmol), digested with phospholipase C, and the resulting DGs were converted to their 1-anthroyl derivatives. Both normal-phase and reversed-phase analysis of this mixture revealed that the phospholipase C hydrolysis of the two phospholipids was equal and complete. The area under the peak was proportional to the amount of these derivatives injected into the HPLC system (Fig. 2A). This enables the addition of molecular species like dipentadecanoyl- or 1-O-hexadecyl-2-pentadecanoyl-*sn*-glycerol-3-phosphocholine or -phosphoethanolamine to be em-

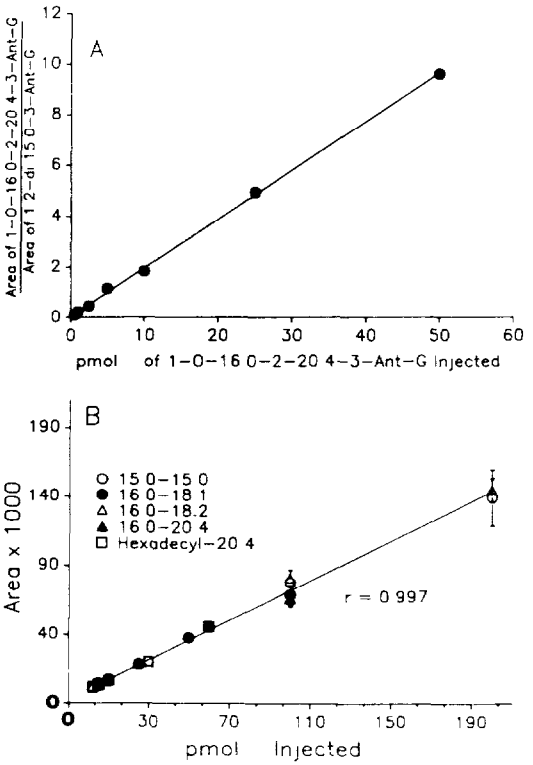

Fig. 2. (A) Standard curve for 1-O-hexadecyl-2-arachidonoyl-3-(1-anthroyl) glycerol (-Ant-G) with 5 pmol of 1,2-dipentadecanoyl-3-(1-anthroyl) glycerol as internal standard. (B) Standard curve for different molecular species of 1,2-diradyl-3-(1-anthroyl) glycerols.

ployed as internal standards for both the quantitation of molecular species and for estimating recoveries after digestion by phospholipase C and derivatization. However, in samples wherein the recoveries after digestion by phospholipase C and derivatization can be monitored by radioactivity, 1,2-dipentadecanoyl-3-[1-anthroyl]-glycerol can be added as an internal standard to the sample following derivatization for routine quantitation of molecular species. Because of the high molar extinction coefficient of the anthroyl group (> 68 000), the detection was linear over a wide range and quantities as low as 0.5 pmol of a sample injected could be detected (Fig. 2A). When the anthroyl derivatives of a number of DG molecular species were analyzed by reversed-

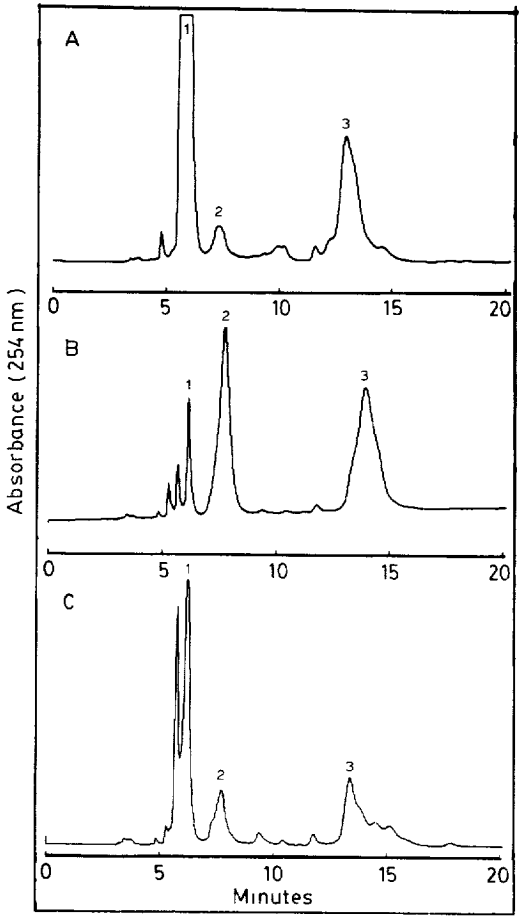

Fig. 3. Normal-phase HPLC separation of phospholipid subclasses from bovine brain ethanolamine phospholipid (A), human PMNL choline phospholipid (B), and human PMNL ethanolamine phospholipid (C). Peaks: 1 = alkenylacyl glycerol; 2 = alkylacyl glycerol; 3 = diacyl-3-[1-anthroyl] glycerol.

phase HPLC, there was a very good correlation (correlation coefficient, $r=0.997$) between the amount of each molecular species injected and the total integration units obtained from the data processor (Fig. 2B).

Normal-phase HPLC of the DG-3-[1-anthroyl] derivatives prepared from biological samples resolved all the three subclasses (Fig. 3). The total amount of each subclass separated by HPLC can be quantitated from the area under each peak. Table I shows the values thus obtained for the three subclasses of ethanolamine phospholipids from bovine brain, and ethanolamine and choline phospholipids from human PMNLs, and these are in good agreement with published values for these samples [13,22]. However, for a bulk separation of the subclasses, TLC was faster than HPLC and here again the three subclasses were well separated with R_F values of 0.52, 0.67, and 0.8 for diacyl, alkylacyl, and alk-1-enylacyl subclasses, respectively. Preliminary results indicate that using similar normal-phase HPLC conditions, the corresponding DG-3-[1-anthroyl] derivatives of platelet activating factor and its acyl analogue can be resolved from each other and well separated from their long-chain acyl analogues.

Fig. 4 shows the composition of molecular species of the individual subclasses from bovine brain ethanolamine phospholipids. Composition and relative distribution of each of the peaks is given in Table II. Identification of the peaks was done by a combination of (a) the retention time using appropriate standards, (b) collecting the peak and analyzing its fatty acid composition by GC, and (c) plotting the logarithm of the relative retention times versus carbon number as previously described [7]. Although the absolute amounts (mol-%) of each molecular species in individual subclasses were not calculated, the relative distribution (expressed as area-%) of each species is very similar to those reported for their acetate [12] and benzoate [13] derivatives.

In conclusion, these studies show that 1-anthroyl nitrile is a new and useful reagent for the analysis of phospholipid subclasses and their molecular species

TABLE I

SUBCLASS COMPOSITION OF ETHANOLAMINE- AND CHOLINE-CONTAINING PHOSPHOLIPIDS FROM BOVINE BRAIN AND HUMAN PMNLs

Phospholipid	Composition (mean ± S.D., $n=3$-4) (area-%)		
	Alkenylacyl	Alkylacyl	Diacyl
Ethanolamine phospholipid (bovine brain)	64.2 ± 2.1	4.8 ± 1.2	31.0 ± 4.3
Ethanolamine phospholipid (human PMNLs)	68.5 ± 4.1	9.3 ± 2.1	22.2 ± 7.3
Choline phospholipid (human PMNLs)	10.1 ± 3.0	45.2 ± 9.0	44.7 ± 5.0

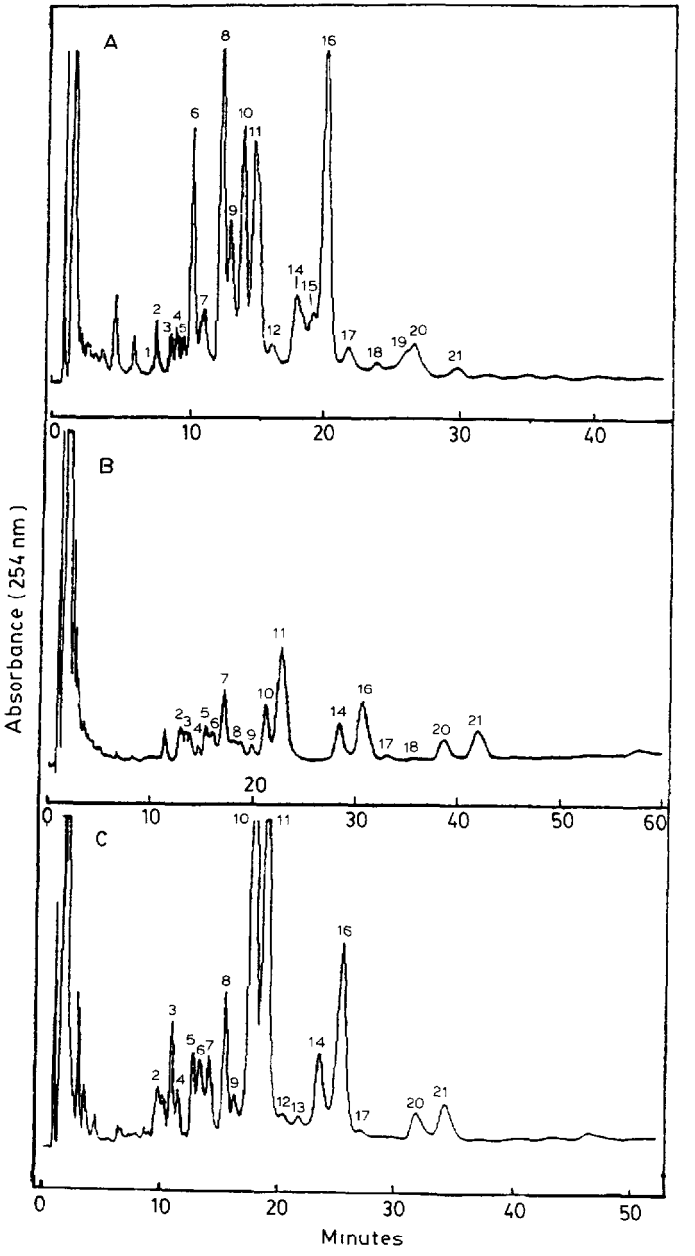

Fig. 4. Reversed-phase HPLC separation of molecular species of bovine brain ethanolamine phospholipid subclasses as their 1,2-diradyl-3-(1-anthroyl) derivatives. (A) Alkenylacyl derivative; (B) alkylacyl derivative; (C) diacyl-(1-anthroyl) derivative.

TABLE II

DISTRIBUTION OF MOLECULAR SPECIES OF DIACYL, ALKYLACYL AND ALKENYL-ACYL SUBCLASSES OF BOVINE BRAIN ETHANOLAMINE PHOSPHOLIPIDS

Peak[a]	Molecular species[b]	Distribution (mean ± S.D., $n=4$) (area-%)		
		Diacyl	Alkylacyl	Alkenylacyl
1	16:0-22 6	Trace[c]	—	Trace
2	18:1-20:4			
	18:1-22:5	2.0 ± 1.9	3.1 ± 1.6	1.1 ± 0.7
3	16:0-20:4	1.3 ± 0.4	2.7 ± 1.0	3.6 ± 0.2
4	16:0-22:5	1.0 ± 0.1	2.4 ± 2.2	1.5 ± 0.1
5	18 1-22:4	0.6 ± 0.2	3.5 ± 0.7	2.7 ± 0.2
6	18·0-22:6	6.7 ± 0.6	2.8 ± 0.7	3.3 ± 0.4
7	16.0-22 4	2.8 ± 0.6	8.3 ± 1.6	3.8 ± 0.4
8	18 0-20.4	11.1 ± 1.5	2.3 ± 1.3	5.5 ± 0.1
9	18:0-22.5	4.6 ± 1.5	1.6 ± 0.4	1.5 ± 0.1
10	18:1-18 1			
	18.0-22 4	13.0 ± 3.5	9.1 ± 0.6	25.7 ± 0.7
11	16·0-18.1	15.8 ± 2.9	30.4 ± 3.1	24.7 ± 1.0
12	16:0-16 0	2.2 ± 1.6	—	Trace
13	17.0-18:1	—	Trace	0.6 ± 0.2
14	18:1-20·1	6.5 ± 1.4		
15	16:0-20.1	1.7 ± 1.3	8.0 ± 0.9	5.9 ± 0.3
16	18:0-18:1	20.6 ± 1.4	15.7 ± 1.8	14.0 ± 0.2
17	16:0-18:0			
	18:0-16:0	1.7 ± 1.3	—	Trace
18	17:0-20:1	1.0 ± 0.7	Trace	—
19	18:1-22:1			
20	18 0-20:1	5.5 ± 0.7	5.2 ± 0.5	2.3 ± 0.1
21	16 0-22:1			
	18.0-18:0	0.8 ± 0.7	8.7 ± 1.5	3.4 ± 0.1

[a]Peak numbers correspond to those in Fig. 4A-C.

[b]Carbon chain length and number of double bonds in sn_1 and sn_2 positions, respectively, are represented (vinyl ether double bond in the alkenyl subclass is not included). Because of the limited samples and lack of internal standards, identity of smaller peaks (< 3 0%) is based solely on their relative retention time and therefore tentative.

[c]Value < 0.5% of total area.

as DG-3-[1-anthroyl] ethers. The method is very sensitive to picomole quantities of phospholipids. The formation of DG-3-[1-anthroyl] ethers is facile and occurs under conditions that do not degrade or rearrange the DG substrate. The presence of the anthroyl moiety does not interfere with the physical characteristics of the diradylanthroyl derivative permitting excellent and predictable chromatographic resolution particularly when compared with the parent phospholipids.

When compared with other chromophores (benzoates and nitrobenzoates) designed for analysis of phospholipids by HPLC [13–15], the anthroyl adduct

is at least five- to ten-fold more sensitive. This is due to the higher extinction coefficient of the anthroyl group: > 68 000 versus ~ 13 000 for the benzoyl group. As a result, an amount as low as 0.5 pmol of a phospholipid molecular species can be measured with a signal-to-noise ratio of about 5:1. For samples containing multiple peaks, about 2 pmol per peak were sufficient for quantitation and gave a signal-to-noise ratio of about 5:1 (e.g. Fig. 4B was obtained by injecting approximately 12 pmol of total material at attenuation 4). Furthermore, because of the fluorescent properties of this adduct, the sensitivity may increase significantly with a fluorescence detection system. Thus, the present method offers a sensitive procedure for a qualitative and quantitative measurement of the levels of phospholipid subclasses and molecular species.

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